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# **Pharmacological Modulation of Lipoprotein Metabolism**

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**Thesis submitted for the degree of Doctor of Philosophy  
in the Faculty of Medicine, University of Glasgow, UK.**

**Submitted June 1992**

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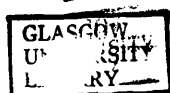
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## Abstract

This thesis has two inter-related aims: to investigate the effects of a series of lipid lowering regimens on the metabolism of the apoB containing lipoproteins and to provide new information on the pathophysiology of human lipoprotein metabolism.

By studying the effects of simvastatin, the resins colestipol and cholestyramine, acipimox and the fibric acid derivatives ciprofibrate and fenofibrate, alone and in selected combinations, it was possible to offer new mechanisms of action for these lipid lowering drugs in patients with primary moderate hypercholesterolaemia. The studies involved the measurement of lipids, lipoproteins and an assessment of either apoB or apo-LDL metabolism at baseline and during the drug therapies. The data obtained from the kinetic studies were analysed using a multicompartmental mathematical modelling strategy. This allowed the computation of rate constants and sub-compartmental masses for each of the apoB containing lipoproteins, which served as a mathematical approximation of apoB or apo-LDL metabolism. Additionally, in the case of simvastatin and fenofibrate, an assessment of the *in vivo* effects of these drugs on cholesterol biosynthesis was made using assays for the cholesterol precursors, mevalonic acid and lathosterol.

The main findings from these studies were a new appreciation for the metabolic heterogeneity that underlies primary moderate hypercholesterolaemia, an increased recognition of the role of the apoB/E receptor in the clearance of LDL precursors as seen in studies with simvastatin and colestipol, formulation of a proposed mechanism for the hypertriglyceridaemia often noted with sequestrant resin therapy and support for the significance of hepatic lipase in IDL-LDL conversion as demonstrated in the study with acipimox. Confirmation of the efficacy of combined lipid lowering therapy is offered, as is a novel mechanism for the enhanced LDL clearance observed with fibrate therapy. The latter involves drug induced changes in the ligand rather than the receptor. Further investigation of the effects of fenofibrate suggested that, unlike simvastatin, this fibrate had no consistent effect on cholesterol biosynthesis. The latter is an important new *in vivo* observation which supports a growing body of *in vitro* work.

This thesis concludes with a discussion of unanswered questions which are of interest both in terms of lipoprotein metabolism and the development of atherosclerosis. New approaches to the study of lipoprotein kinetics are suggested involving the application of stable isotope technology, and an hypothesis, based on kinetic and structural observations, that the direct synthesis of LDL apoB is linked more closely with the larger, lighter LDL subfractions, is stated.

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## Acknowledgements

The dwarf sees farther than the giant, when he has the giant's shoulder to mount...

Samuel T. Coleridge, *The Friend*, 1772 1834.

Just as Coleridge's dwarf was only able to glimpse a farther horizon with the help of another, I have only risen above the parapet of my own inexperience and ineptitude by standing on the shoulders of a veritable human pyramid. The following list acknowledges those, whose contributions of various kinds made the completion of this work possible.

*My Patients*, for placing their trust in me and always giving more than was asked.

*James Shepherd*, my supervisor, for making all things possible and expanding my imagination of what might be possible.

*Christopher Packard*, for always having an open door and tolerating an intellect unequal to his own.

*Shona Collins*, for providing technical assistance with the VLDL turnovers.

*Elizabeth Murray*, for continuing with this technical assistance with the VLDL turnovers and keeping calm when all about her were falling apart.

*Grace Lindsay*, for providing nursing support in the patient studies, mathematical support in the SAAM modelling, and moral support at all times.

*Thomas Demant*, for teaching me the practicalities of the VLDL turnover technique, and for sharing his vast knowledge of the subject.

*Philip Stewart*, for help in setting up and running of the Lathosterol assay, and for knowing where everything was in the laboratory.

*Bruce Griffin*, for performing the LDL subfraction analyses and for being a source of constant encouragement.

*Muriel Caslake*, for performing the LDL subfraction analyses and for technical and modelling support with the LDL turnovers.

*Dorothy Bedford*, for performing apoE phenotypes and HDL subfractions on the patients, and for teaching me how to perform the laboratory work associated with a VLDL turnover.

*Linda McCusker*, for performing apoE phenotypes, Lp(a) and HDL subfractions.

*Kenneth Lindsay*, for assisting with the input of data into the university VAX.

*Tim Watson*, for valuable discussion and serving as an example of how its done.

*Graham Warwick* for valuable discussions and for clinical assistance.

*Dilys Freeman*, for her constant enthusiasm and her belief that all would come right.

*Herman Kempen & Tatu Miettinen*, for their valuable correspondence on the lathosterol assay.

*Anne Bell* & the technical staff of the routine lipid section at Glasgow Royal Infirmary, Department of Biochemistry, for performing all the  $\beta$  quantas and all the compositional assays.

*Roger Illingworth, Anu Pappu, & Shannon Magennis* of OHSU in Portland Oregon, for inviting me to their labortaory and sharing with me their Mevalonate Assay.

*George Popjak*, for his kindness in Los Angeles and reminding me on a sunny day what science was all about.

*Ross Lorimer, David Ballantyne, & Barry Vallance* for allowing me to study their patients.

*Max Nanjiani*, for performing slit lamp examination of those patients receiving simvastatin.

*DC Thomson & Co* , for allowing me to use their cartoon character, Oor Wullie in a patient information booklet explaining the turnover protocol.

*MSD, Upjohn, Farmitalia, Fournier, Bristol-Myers Squibb, & Sanofi-Winthrop* for gifts of their drugs.

At the bottom and bearing the weight of it all has been my foundation, Moira, my wife. Without her and all that she has done and all that she has had to forego, this edifice would have been built on shifting sand.

## *Author's Declaration*

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

Allan Gaw, June 1992

*Dedication*

I believe all research must look to the future for its justification. Our labours and any success they may bring today are our legacy for tomorrow. Like any father my tomorrow is that of my children and it is to them, Stephen and Alexandra, that I dedicate this work.

## Chapter 1 Introduction

These are the springs of man's existence: from them spread throughout his body those rivers with which his mortal habitation is irrigated, those rivers which bring life to man as well, for if ever they dry up, then man dies.

Hippocrates, *De Corde* c 260 BC.

### 1.1 Clinical Significance of Lipoprotein Research

Even in antiquity, the importance of cardiovascular function and integrity were well recognised. Today the words of Hippocrates have greater relevance than ever before, for coronary heart disease (CHD) is the leading cause of death in the UK and the developed world. In 1989 CHD accounted for 26% of the annual all-cause mortality in England and Wales (Office of Population Censuses and Surveys 1990) and the annual cost of this disease to the health service has been estimated at approximately £500 million (Office of Health Economics 1990).

The aetiology of coronary heart disease has been the subject of intense study over the last few decades and many contributory or risk factors have been identified, and these are well summarised by Isles & Hole (1991). Much attention has focussed on the three principal and modifiable risk factors viz. smoking, hypertension and hypercholesterolaemia. These risk factors have been identified repeatedly throughout the literature (Kannel *et al* 1971, Shaper *et al* 1981, Multiple Risk Factor Intervention Trial Research Group 1982) and are now well accepted. Their modification on a population basis, particularly by dietary means, is advocated by several agencies including the British Government (Committee on Medical Aspects of Food Policy 1984 & 1991) and the World Health Organization (1990). That changes can be made at all, and in a relatively short time, is clear when we examine the worldwide trends in age-adjusted death rates for CHD in men between the years 1970 and 1985 (Uemura & Pisa 1988). The US leads this field, having achieved an almost 50% reduction in deaths from CHD in this period while countries such as Romania and Poland have shown marked increases of more than 70%. Scotland,



in the same 15 year period, has shown a fall of approximately 10% but remains at or near the top of the international league table of male CHD mortality (Marmot 1988). Scotland does not, however, have the highest mean total cholesterol levels in the world. Of the 20 countries surveyed in the on-going WHO MONICA project (1988) Scotland has the joint third highest (6.2 mmol. L<sup>-1</sup> in males aged 35-64 yrs), but revealingly led the world in the prevalence of multiple CHD risk factors. This point re-iterates the fact that CHD is a multifactorial disease.

As this thesis takes as its basic premises, firstly, that hypercholesterolaemia is a major risk factor for CHD and secondly that modification of the plasma cholesterol level is worthy of pharmacological intervention when dietary management fails, we must further examine the evidence for these.

The putative role of hyperlipidaemia in the development of atherosclerosis goes back almost 150 years to the work of Vogel (1845) in Leipzig. It was he who first noted the presence of cholesterol in atheromatous tissue. The term atherosclerosis itself was coined by Marchand in c1860 (quoted by Aschoff 1924) but it was not until the early years of the twentieth century that the association between dietary cholesterol and atherosclerotic lesions was confirmed experimentally. Anitschkow (1913) in St. Petersburg fed egg yolk to rabbits and observed the development of lesions in their aortae, identical to the atherosclerotic lesions in man. As this work progressed, Anitschkow (1915) became convinced only two years later that, 'there can be no atheroma without cholesterol'.

From such beginnings an impressive edifice of epidemiological research has been built, culminating in incontrovertible evidence for the recognition of hyperlipidaemia as an important causative factor in the development of atherosclerosis. Because hyperlipidaemia may be readily corrected, in most cases, by dietary and pharmacological means, it has been the focus of much clinical attention.

The Lipid Research Clinics Coronary Primary Prevention Trial (Lipid Research Clinics Program 1984a & 1984b) and Helsinki Heart Study (Frick *et al* 1987), clearly showed that the lowering of plasma cholesterol levels brings benefit in terms of reduction of coronary morbidity. Such findings coupled with advances in therapeutics and biotechnology have detonated an explosion of interest and activity within the scientific and medical communities in the last five years.

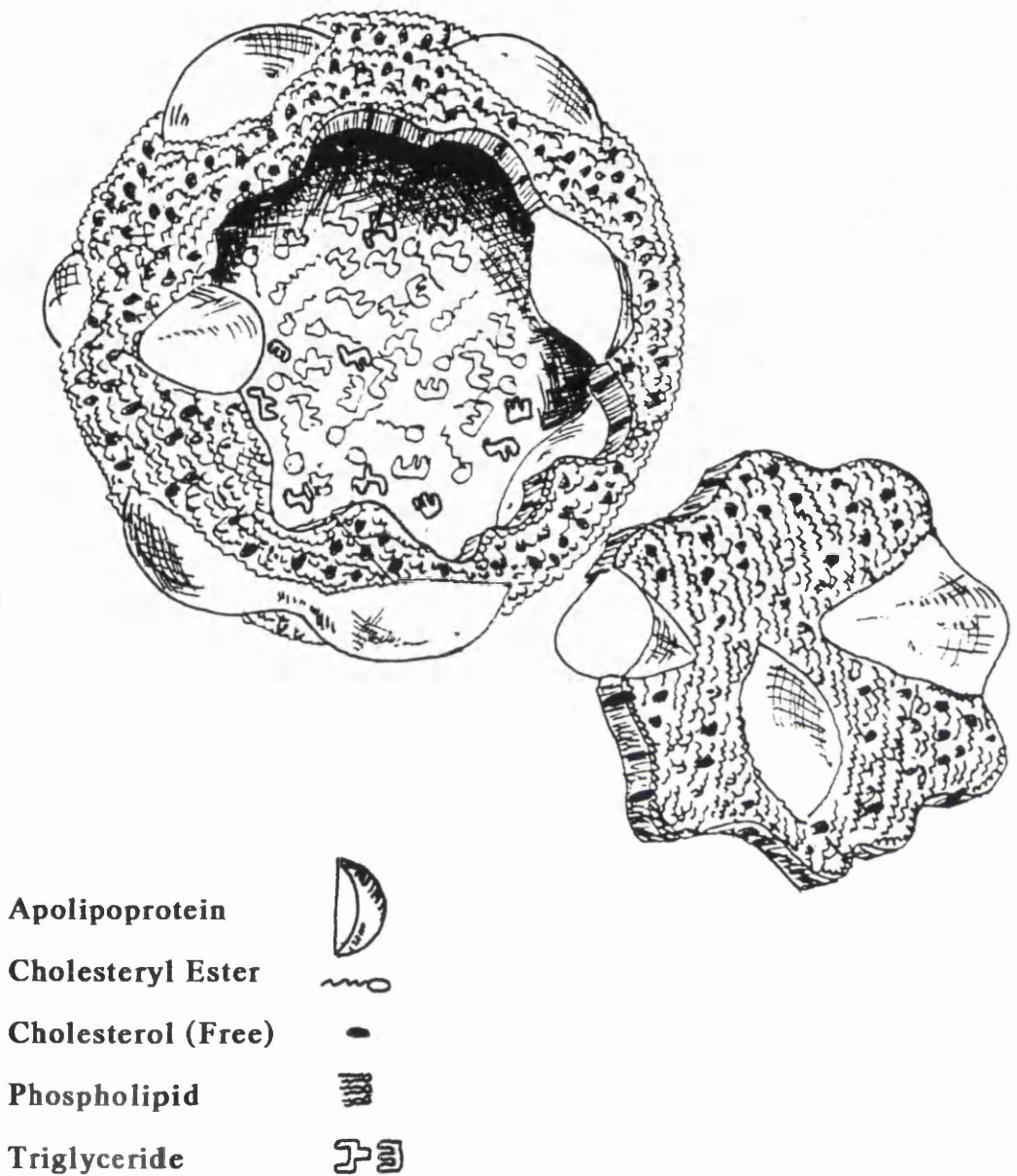
Our ability to modulate the cholesterol and lipoprotein levels in the plasma, and in turn the coronary heart disease risk of an individual, is a reflection of the efforts devoted to this subject over the past twenty years, and depends largely on our understanding of lipoprotein metabolism. With the control of lipoprotein metabolism comes the control, at least in part, of the development of atheroma. There follows a description of the structure and function of the plasma lipoproteins, an overview of normal and deranged lipoprotein metabolism, a discussion of the methods employed to study lipoprotein synthesis, interconversions and catabolism, and a review of the different forms of hypolipidaemic therapies now in clinical use.

## **1.2 Lipids, Lipoproteins, and their Metabolism.**

Lipids are important sources of energy, of synthetic precursors, and cellular components. Because of this a complex system has evolved to solve the problem of transporting lipids around the body in the aqueous environment of the plasma. This system depends on the packaging of neutral lipids (cholesteryl esters and triglyceride) with specific proteins and amphipathic lipids (phospholipids and cholesterol) to create multimolecular particles called lipoproteins that are readily miscible with water (figure 1). The major organs involved in lipid metabolism are the intestine and the liver. Together these organs are responsible for the majority of lipoprotein synthesis and catabolism. Regulation of lipid transport is exerted by several means: apolipoproteins with specific signalling and cofactor functions, specific cell-surface lipoprotein receptors, intravascular lipolytic enzymes and transfer proteins that act in concert to maintain cholesterol and triglyceride homeostasis. Malfunction of these regulatory factors may cause or contribute to the development of dyslipidaemia and in turn atherosclerosis. A simple overview of lipoprotein metabolism illustrating the roles that each plays in lipid transport is illustrated in figure 2.

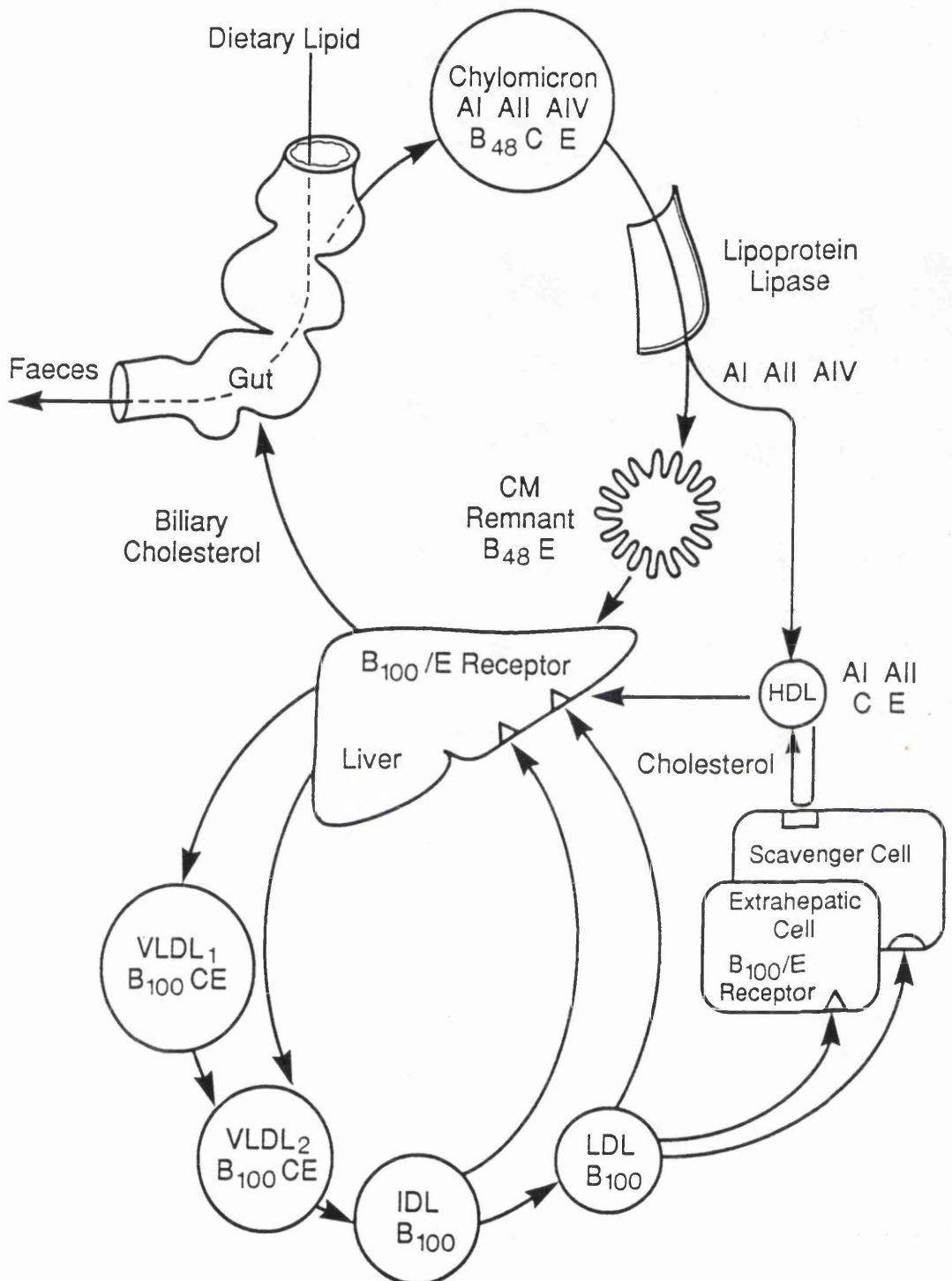
### **1.2.1 Classification and Nomenclature of Plasma Lipoproteins**

The resolution of the plasma lipoproteins was made possible largely due to the use of the ultracentrifuge in the 1950s (Gofman *et al* 1950a, Turner *et al* 1951). The most widely used system of nomenclature defines five main classes of lipoprotein based on their hydrated density (g. mL<sup>-1</sup>) (Mills, Lane & Weech 1984). Lipoproteins have also been defined by their electrophoretic mobility, particle size, apolipoprotein composition and flotation rate (Mills *et al* 1984). The latter is commonly quoted and merits further explanation. In this system the lipoproteins



**Figure 1.** Impression of Lipoprotein Structure.

Lipoprotein particles are multi-molecular complexes of roughly spherical shape. The example illustrated above shows the main elements of all lipoprotein classes. Each has a surface monolayer of phospholipid, cholesterol and the hydrophilic domains of the constituent apolipoproteins. In this diagram, the exploded section reveals the hydrophobic core of the particle, which contains triglyceride and cholesteryl esters. The hydrophobic domains of the apolipoproteins and the phospholipid tails are thought to abut on this lipid cargo. The size and composition of a lipoprotein particle vary from one class to another. Although the precise apolipoprotein configuration illustrated here is imaginary, the molecular scale of this diagram would suggest a particle diameter of 50nm, approximating its structure most closely to that of VLDL<sub>1</sub>.



**Figure 2.** Overview of Lipoprotein Metabolism

Following its absorption, dietary fat is packaged into large, triglyceride-rich chylomicron particles within the enterocyte and secreted into the circulation. There, lipolysis reduces the particle's triglyceride core and makes redundant part of its surface coat, which is shed to high density lipoprotein (HDL). The remnants produced in the process are rapidly assimilated by a receptor-mediated mechanism in the liver. In the fasting state, very low density lipoprotein (VLDL) replace chylomicrons as the major triglyceride transporters and the liver dominates lipoprotein metabolism. Cholesterol and triglyceride elaborated in this organ, are released into the plasma where they are subject to tissue lipolysis. This degrades them to low density lipoprotein (LDL) via an intermediate species (IDL). The LDL is removed by receptors present on the liver and peripheral tissues. When these are saturated, alternative scavenger receptor pathways become dominant.

are defined by their rate of flotation through a salt solution of fixed density in the analytical ultracentrifuge. For lipoproteins of density less than  $1.063 \text{ g. mL}^{-1}$  the salt solution used is NaCl d.  $1.063 \text{ g. mL}^{-1}$ , while the high density lipoproteins are defined using a density solution of  $1.210 \text{ g. mL}^{-1}$ . All densities are defined at  $26^\circ\text{C}$  and the units used are Svedberg units ( $S_f$ ) where one  $S_f$  unit =  $10^{-13} \text{ cm. s}^{-1}$ .  $\text{dyne}^{-1} \cdot \text{g}^{-1}$  at  $26^\circ\text{C}$ . Both systems are very flexible; while five main lipoprotein classes are traditionally defined, any lipoprotein preparation that does not fit these established windows may be characterized by its limiting densities or flotation rate interval. The physical properties of the plasma lipoproteins are summarised in table 1, and their chemical compositions are shown in table 2. Each lipoprotein class exhibits a unique structure, function, and metabolism as described below in sections 1.2.2-1.2.11.

### **1.2.2 Structure and function of Lipoprotein classes and subgroups.**

The structure of the different lipoprotein classes, their lipid composition and their apolipoprotein components are inextricably linked to their function and to their metabolism in the body. As such it is artificial to separate the two and structure and function will be discussed together in the subsequent sections.

### **1.2.3 Intestinal Lipid Metabolism**

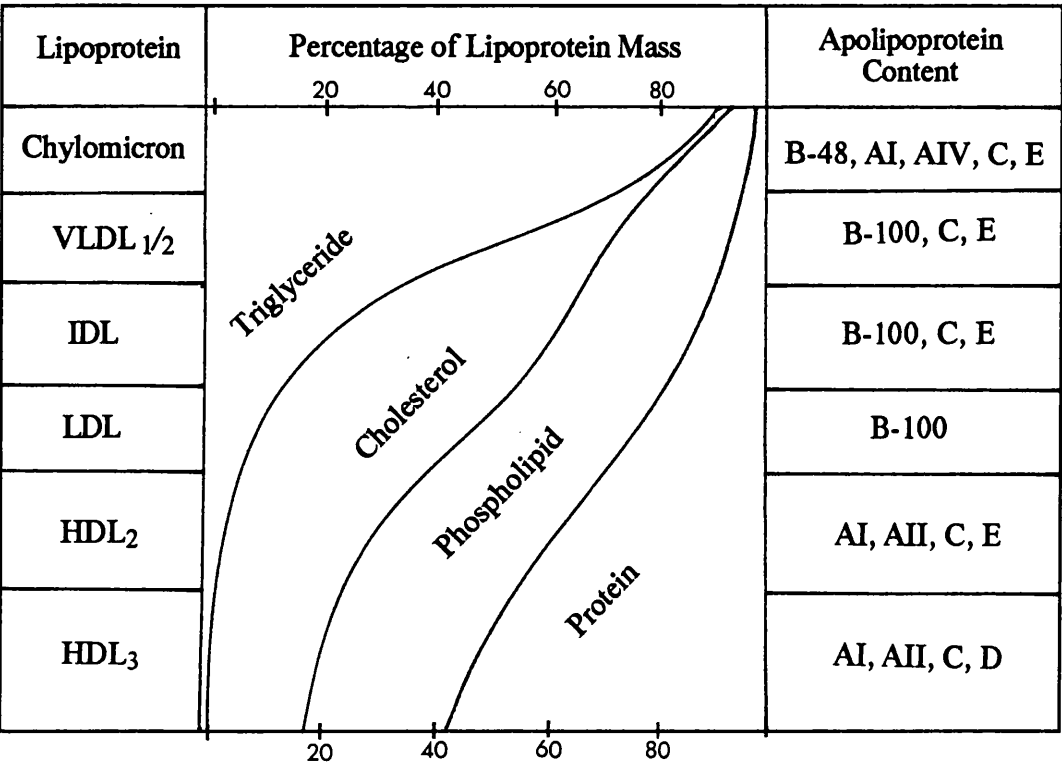
Daily we ingest about 0.5g of cholesterol (as free and esterified sterol) and 100g of triglyceride. Digestive enzymes in the intestinal lumen hydrolyze the lipid esters, releasing free cholesterol, fatty acids and mono- and diglycerides. These amphipathic molecules form water-soluble micelles that carry the lipid to absorptive sites in the duodenum (Danielson & Sjoval 1975). Under normal circumstances, triglyceride absorption is virtually complete. However, only about 50% of the cholesterol is taken up, the remainder being lost in the faeces (Norum *et al* 1983). Within the enterocyte, absorbed cholesterol is rapidly re-esterified by the action of the membrane bound cytoplasmic enzyme acyl Coenzyme A: cholesterol acyltransferase (ACAT) (Norum *et al* 1983) and packaged with reconstituted triglyceride into large, lipid-rich chylomicron particles containing about 1% protein. These appear in abundance in the intestinal mucosal cells following a meal and are secreted into lacteals within the wall of the small bowel. The availability of a specific structural apolipoprotein known as apolipoprotein B (apoB) is a prerequisite for the production of such particles. Subjects with the inherited disorder known as abetalipoproteinaemia (Kane & Havel 1989) fail to secrete the

Table 1. The Physical Properties of the Plasma Lipoprotein Classes

Lipoprotein	Density Range g. mL <sup>-1</sup>	Sf at 26°C & 1.063 g. mL <sup>-1</sup>	Diameter nm	Corresponding Electrophoretic Mobility
Chylomicron	<0.94	>400	75-1200	Origin
VLDL <sub>1/2</sub>	0.94-1.006	60-400 20-60	30-80	Pre-β
IDL	1.006-1.019	12-20	25-35	Slow pre-β
LDL	1.019-1.063	0-12	18-25	β
HDL <sub>2</sub>	1.063-1.125	-	10-15	α
HDL <sub>3</sub>	1.125-1.210	-	5-10	α

Physical data taken from Mills, Lane & Weech (1984)

Table 2. The Chemical Composition of the Plasma Lipoprotein Classes.



Compositional data taken from Mills, Lane & Weech (1984) and from own data.

chylomicrons from their enterocytes following a fatty meal. The condition results in failure to thrive in infancy and is associated with specific retinal and erythrocyte defects that are progressive and derive ultimately from defective absorption of fat soluble vitamins.

ApoB found in chylomicrons is a modified, lower molecular weight form of that synthesised by the liver and secreted with VLDL. The former is termed apoB-48 and the latter apoB-100 in the nomenclature of Kane, Hardman & Pauls (1980). It has recently been noted (Levy *et al* 1990) that the gut also produces apoB-100 although this does not appear in chylomicrons. Other proteins are also present on the chylomicron surface. Some (apo AI, AII, AIV and possibly C) are elaborated by the gut and secreted with the chylomicron (Green & Glickman 1981) while additional peptides are acquired from high density lipoprotein (HDL) in interstitial fluid. The latter include apoC and apoE, both of which participate in subsequent processing of the particle (Green & Glickman 1981; Imaizumi, Fainaru & Havel 1978; Green *et al* 1979).

Several rapid changes take place in the chylomicron when it enters the plasma. Within the capillary beds of skeletal muscle and adipose tissue it is exposed to the lipolytic action of endothelium-bound lipoprotein lipase. This enzyme hydrolyzes its triglyceride core, releasing free fatty acids and mono- and diglycerides for energy production or storage (Nilsson-Ehle, Garfunkel & Schotz 1980). Further delipidation results in the generation of so-called 'chylomicron remnants'. These particles are structurally distinct from the parent chylomicron (Mjos *et al* 1975), not only because they are the products of the degradation process outlined above but also because they acquire lipid, particularly cholesteryl ester, from denser lipoproteins like LDL and HDL, facilitated by cholesteryl ester transfer protein (CETP). Zilversmit (1984) demonstrated that this cholesteryl ester is exchanged for triglyceride transferred in the opposite direction, i.e., into LDL and HDL. Deficiency of CETP activity as described by Eto *et al* (1990) results in markedly elevated plasma levels of HDL. The extent of this process, which effectively enriches chylomicron remnants in cholesteryl esters, is governed by the residence time of the chylomicron in the circulation. Rapid chylomicron clearance resulting from highly efficient lipolysis, gives the particle little opportunity to acquire cholesteryl ester. Besides these alterations in its hydrophobic core, the surface coat of the remnant shows substantial differences from that of its parent. All the apoB initially present in the particle is retained, but the relative amounts of the other proteins are altered. ApoAI and apoAII are transferred into the HDL density range

(Schaefer, Jenkins & Brewer 1978; Tall *et al* 1979) while apoAIV, upon release from the chylomicron, seems to exist free in the plasma since its affinity for other lipoproteins is low (Tall *et al* 1979). As noted above the C protein content initially rises as a result of transfer from HDL (Havel, Kane & Kashyap 1973). This is of particular significance with regard to apoCII since this is an obligatory cofactor for lipoprotein lipase, increasing the particle's affinity for the enzyme. The role of apoCIII, the major C peptide on the chylomicron surface, is less clear although it may be responsible for regulating the lipolysis of the particle (Ginsberg *et al* 1986) or delaying its hepatic clearance (Windler, Chao & Havel 1980). As the chylomicron progresses down the lipolytic cascade the C proteins are transferred gradually back into the reservoir within HDL. ApoE, which is acquired by the particle simultaneously with apoC, is not involved in lipolysis (Blum 1982), but thereafter plays a role in triggering hepatic uptake of the remnant particle (Mahley & Innerarity 1983).

The liver is responsible for the efficient and rapid clearance of cholesteryl ester rich chylomicron remnants from the circulation. Hepatic parenchymal cells contain, on their surfaces, receptors that recognise and bind to particular charged domains of the remnant's apoE (Brown & Goldstein 1987). This interaction results in the delivery of the particle to lysosomal degradation within the hepatocyte. The key role played by apoE in this process is evident from studies performed on individuals who express a mutant form of the E protein (apoE2) in which the conformation of the receptor-binding region is altered. They exhibit delayed clearance of the remnants from the bloodstream (Gregg *et al* 1981). The metabolism of chylomicrons may be regarded as a bi-functional process, involving the delivery of dietary triglyceride to skeletal muscle and adipose tissue, and of cholesterol to the liver. The activity of lipoprotein lipase in a tissue is constantly changing and is under hormonal control. Thus the enzyme has some of the properties of a membrane receptor in determining the direction of triglyceride flow.

#### 1.2.4 Hepatic Lipid Metabolism

Although the liver has the capacity to make all the cholesterol and triglyceride that it needs, preformed components that are available either from the diet or from adipose tissue via fatty acid/albumin transport are preferred. Mobilization of depot fat in adipose tissue is under humoral control by agents such as adrenaline (Krebs & Beavo 1979). The fatty acids released from the adipocyte into the circulation are rapidly extracted by many tissues including the liver, which is able to use them for



energy and as substrates for the synthesis of phospholipids and triglyceride. When the availability of these substrates is limited, the liver can generate fatty acid from small molecular weight precursors (Barter & Kevin 1972). Similarly a cholesterol deficit can be met by endogenous synthesis, following activation of the rate limiting enzyme 3-hydroxy 3-methylglutaryl Coenzyme A reductase (HMG CoA reductase) (Goldstein & Brown 1984).

Lipids synthesized in the liver have several fates (Norum *et al* 1983; Packard & Shepherd 1986). First, a significant proportion of the cholesterol and triglyceride is secreted in VLDL, the major vehicle for endogenous triglyceride transport. Secondly, lipid surplus to requirements may be stored, temporarily, as cytoplasmic oil droplets within the hepatocyte. And, thirdly, the liver cell has the unique ability (Dietschy & Wilson 1970) to eliminate cholesterol into the bile, either unchanged or following oxidation to bile acids. Large (gram) quantities of lipid flux through these pathways each day and so it is imperative for the hepatocellular economy that the mechanisms involved are precisely interregulated. This is achieved by the coordinated control of certain key enzymes whose activities appear in turn to be modulated by bile acids. These compounds, produced by the liver and secreted into the gut to aid digestion, are reabsorbed in the terminal ileum and extracted from the portal blood by the liver. Their return to their site of origin in this 'enterohepatic circulation' produces several important effects (Angelin, Bjorkhem & Einarsson 1986), viz:

- a. Cholesterol 7 $\alpha$  hydroxylase, the rate limiting enzyme in bile acid synthesis, is downregulated.
- b. HMG CoA reductase, the enzyme that governs cholesterol production, is also suppressed, although whether this is a direct or indirect effect of bile acids is a matter of controversy.
- c. Phosphatidic acid phosphatase, which stands at the fork between the pathways of triglyceride and phospholipid synthesis, is inhibited, favouring production of the latter.

The net result of these regulatory events is to ensure that, as bile acids flow back to the liver, they suppress their *de novo* synthesis while stimulating adequate secretion of the co-emulsifier, lecithin into the bile. The enterohepatic flow of bile acids follows the cycle of dietary intake, and so strong diurnal rhythms are induced in these control mechanisms as evident from detailed studies of rats kept under conditions of controlled lighting and feeding (Danielson & Sjovall 1975; Dietschy

& Wilson 1970; Myant & Mitropoulos 1977). When food is administered during the dark period it triggers a release of bile acids from the hepatobiliary tree. This produces a peak in the synthesis of hepatic triglyceride, cholesterol and bile acids, and secretion of VLDL into the blood stream also reaches a peak (Goh & Heimberg 1979). The above phenomena are reversed in the middle of the light period when the liver is replete with recycled bile acids. However, although this scheme provides a plausible explanation for the laboratory observations, it is incomplete since even continuously fasted animals show periodicity in hepatic synthesis (Myant & Mitropoulos 1977); and in man such diurnal rhythms are less well documented, although there is some good evidence to indicate that they occur (Parker *et al* 1982).

It is clear from the above argument that medical or surgical interruption of the enterohepatic circulation of bile acids will have profound effects on hepatic lipid metabolism. Such therapeutic manoeuvres can therefore be used to advantage in the management of hypercholesterolaemia.

### 1.2.5 Metabolism of ApoB Containing Lipoproteins

In the post absorptive state, VLDL replaces the chylomicron as the main vehicle of triglyceride transport in the plasma. ApoB is integral to the particle and essential for its normal secretion. Apo B in VLDL is, as noted above, the larger molecular weight apoB-100, but shares a number of antigenic sites with the gut-derived apoB-48. The B-100 protein has now been sequenced using recombinant DNA technology (Knott *et al* 1985; Carlsson *et al* 1985; Chan *et al* 1985). It is a huge protein of 4336 amino acid residues that has many repeat sequences. Following synthesis in the rough endoplasmic reticulum, the protein associates with a triglyceride/phospholipid droplet in its passage to the Golgi apparatus where it becomes glycosylated before being secreted (Hamilton 1983). Examination of VLDL particles isolated from purified Golgi membranes has shown that they are heterogeneous, encompassing a size range from 40-70nm. They therefore do not have a stoichiometrically definable structure (Hamilton 1983). This information accords with complementary metabolic evidence that the liver has the capacity to elaborate a range of VLDL particles whose size and lipid composition may vary in response to changes in nutritional status. Besides apoB-100, nascent VLDL particles contain a few molecules of apoC and apoE that are augmented by transfer from HDL when the lipoprotein meets the interstitial fluid.

Triglyceride-rich VLDL enter a metabolic cascade similar to that described for chylomicrons, and indeed both compete for the same lipolytic sites in skeletal muscle and adipose tissue capillary beds. Studies designed to examine the metabolism of VLDL have focussed largely on its B protein moiety. Early work with radioiodinated VLDL of density less than  $1.006 \text{ g. mL}^{-1}$  (Berman *et al* 1978) indicated that apoB was transferred through an intermediate lipoprotein fraction, IDL to LDL. In the process the particle's core is hydrolyzed by lipoprotein lipase (Nilsson-Ehle *et al* 1980), a reaction that requires the participation of apoCII on the particle. Again, in parallel with chylomicron metabolism, cholesteryl esters (Eisenberg 1985) are acquired from other lipoproteins (principally HDL) by exchange, while surface coat apoC is transferred in the opposite direction (Berman *et al* 1978). LDL therefore represents a 'remnant' of VLDL catabolism in which the triglyceride core is virtually eliminated and apoB is the sole protein component. In most subjects, whether normolipaemic or not, the rate of synthesis of apoB into VLDL (Janus *et al* 1980) exceeds that into LDL. Not all VLDL particles are therefore destined to complete their journey down the delipidation cascade to LDL. This phenomenon has been examined in detail (Shepherd *et al* 1984b, Packard *et al* 1984) using procedures that permit fractionation of the VLDL spectrum in order to follow the fates of particles of a narrower compositional range. The evidence that emerged indicated that lipolysis of large VLDL (Sf 60-400) generated remnants of Sf 12-60 (i.e., within the small VLDL/IDL flotation interval), most of which were removed directly from the plasma without appearing in LDL. The latter seemed to come from rapid and quantitative transformation of small VLDL particles that had been secreted directly by the liver.

Both of the major plasma lipolytic enzymes, lipoprotein lipase and hepatic lipase have been implicated in the metabolism of apoB-100 containing particles (Nicoll & Lewis 1980). It is known that the affinity of lipoprotein lipase is greater for large, triglyceride-rich particles than for smaller remnants, whereas hepatic lipase, which expresses both triglyceride lipase and phospholipase activities, seems to favour smaller VLDL and IDL particles. The complementary roles of these lipases are perhaps best exemplified in studies of patients who, for genetic reasons (Carlson, Holmqvist & Nilsson-Ehle 1986; Demant *et al* 1988), lack the enzymes, or in animal studies where enzyme activity has been inhibited using antibodies (Behr *et al* 1981; Goldberg *et al* 1982). Under such conditions, absence of lipoprotein lipase results in accumulation of particles of flotation rate greater than Sf 100 that contain both apoB-100 and apoB-48 (i.e., of gut and liver origin, respectively). Smaller, denser apoB containing lipoproteins virtually disappear from the plasma.

Conversely, antibody induced inhibition of hepatic lipase in cynomolgus monkeys (Goldberg *et al* 1982) causes the accumulation of smaller VLDL and IDL; and LDL becomes relatively enriched in triglyceride. Similarly, individuals with hepatic lipase deficiency express high plasma concentrations of small VLDL and IDL that are accompanied by reduced circulating levels of LDL (Demant *et al* 1988).

The product of the lipolytic cascade, LDL, has been recognised for some years as structurally heterogeneous in patients with hypertriglyceridaemia (Hammond *et al* 1977; Fisher 1983), and more recently has been found to exist in the plasma of all individuals as a group of discrete but overlapping particle populations or subfractions (Shen *et al* 1981; Krauss & Burke 1982). These subfractions have traditionally been separated on the basis of hydrated density and particle size, using density gradient ultracentrifugation and gradient gel electrophoresis respectively, and are defined as LDL-I to LDL-IV (Krauss 1987a). Gofman and his coworkers were the first to demonstrate that LDL is heterogeneous in the analytical ultracentrifuge and to suggest that certain subfractions may be more closely associated with CHD than others (Gofman *et al* 1950b). These early observations have now been substantiated in a series of cross-sectional studies that have revealed a constellation of abnormalities in LDL structure and concentration that lead to increased risk of CHD (Crouse *et al* 1985; Musliner & Krauss 1988). A predominance of small, dense LDL in combination with a raised triglyceride and low levels of HDL has been associated with a three-fold increase in risk of myocardial infarction (Austin *et al* 1988). These changes have been collectively described as an atherogenic lipoprotein phenotype or ALP (Austin *et al* 1990b). There is strong evidence to suggest that ALP, and more specifically the distribution of LDL subfractions, is influenced by a major gene (Austin & Krauss 1986; Austin *et al* 1990a). However, the profile is also subject to the environmental effects of diet and drugs (Griffin *et al* 1992).

#### 1.2.6 Metabolism of ApoA Containing Lipoproteins

The plasma lipoprotein spectrum also encompasses particles whose main protein component is apoA. They lie in the density interval 1.063-1.210 g. mL<sup>-1</sup> and are the smallest of the lipoproteins, having a molecular weight not much different from plasma protein components such as  $\alpha_2$ -macroglobulin. A typical HDL particle is about 7nm in diameter and almost 3000 times smaller than the average VLDL particle in terms of volume. HDL is, therefore, the smallest of the lipoproteins yet

the most numerous, being a heterogeneous mixture of particles endowed with a diversity of metabolic properties.

HDL represents an amalgam of diverse components that come together following:

- a. direct secretion by the liver and intestine;
- b. transfer from other lipoproteins;
- c. transfer from peripheral tissues.

The major HDL proteins, apoAI and apoAII are elaborated in precursor form in the liver and intestine. Estimates of the contribution from each of these organs, at least in rats, suggest equal sharing of synthesis (Wu & Windmueller 1979). Certainly, intestinal apoA production is prodigious as evidenced from its copious appearance in the urine of chyluric patients (Green *et al* 1979). Some of the protein is secreted with chylomicrons and appears in the HDL density interval following lipolysis of these particles (Green & Glickman 1981). Consequently after a fatty meal the levels of apoAI and apoAII in the plasma rise. In addition, the intestine is capable of elaborating and secreting small apoA containing particles directly into the HDL density interval (Green, Tall & Glickman 1978), a process that is continuous even in the fasting state. Studies using perfused intestine preparations have shown that under conditions where lecithin: cholesterol acyltransferase (LCAT) activity is blocked, a discoidal particle is produced, rich in free cholesterol and phospholipid and with apoA arranged around its circumference. Particles with a similar structure are also isolable from lymph (Dory *et al* 1985), a body fluid that is naturally low in LCAT activity. Exposure of such particles to the enzymatic and transfer activities of plasma transforms them into mature spherical HDL (Dory *et al* 1983). The apoA proteins lost from the surface of chylomicrons during lipolysis are thought also to appear initially in bilayer discs that undergo rapid conversion in the plasma to mature HDL spheres (Tall & Small 1978). Electron micrographic studies have shown that lipolysis, by reducing the core volume of the chylomicron renders some of its surface coat redundant. The surface monolayer responds by folding upon itself to form a frond-like bilayer that, shed into the plasma, comes under the influence of LCAT and is converted to mature HDL. The central role of LCAT in the maturation of HDL is evident from studies of those rare patients (Norum 1984) who present with an inherited deficiency of the enzyme. Their HDL circulates as bilayer discs that readily undergo transformation *in vitro* to normal HDL with the addition of LCAT.

As noted above, the liver and the intestine share equally in the synthesis of HDL. Perfusion studies in rats have shown that the primary hepatic secretion product is again discoidal (Hamilton *et al* 1976) and rich in both apoAI and apoE. Exposure to LCAT induces its rapid conversion to HDL. In this process, apoAI is retained while E is lost to lipoproteins of lower density (Norum 1984).

The action of LCAT on discoidal HDL triggers a change in the disposition of cholesterol within the particle. The enzyme catalyzes the transfer of a fatty acid from lecithin to the hydroxyl residue on cholesterol, leading to the generation of lysolecithin and cholesteryl ester (Norum 1984). The polarities of these products are fundamentally different from those of their precursors. Lysolecithin is more hydrophilic and dissociates readily from the lipoprotein into the aqueous environment. On the other hand, esterification of the sterol increases its hydrophobicity and causes it to partition into the nonpolar interior of the particle. The surface site vacated by the sterol is then available to accept additional cholesterol molecules either from other lipoproteins or from cell surfaces. Thus, LCAT has a dual action: it facilitates the sequestration of cholesterol within the hydrophobic core of HDL, generating in the process a chemical potential gradient that leads to continued uptake of the sterol. Even mature HDL particles have a free cholesterol/phospholipid ratio that is less than that of VLDL or LDL. Therefore it is not surprising that HDL is a good acceptor of tissue cholesterol while VLDL and LDL are not. Acquisition of cholesterol by HDL proceeds until the nascent disc is fully transformed into a pseudomicellar spherical structure. Obviously, this process would be constrained by the size of the HDL particles were it not possible for the sterol ester to undergo transfer to less dense lipoproteins facilitated by a neutral lipid transfer protein. This protein exchanges the cholesteryl ester in the core of HDL for triglyceride acquired from VLDL or chylomicrons. The triglyceride enriched HDL then becomes more susceptible to intravascular lipolysis, which renders it smaller and denser (Deckelbaum, Olivecrona & Eisenberg 1984). Thus the size of circulating HDL depends on the balance between the opposing forces of cholesterol esterification, which increases particle size, and lipolytic digestion, which reduces it. A recent report by Cheung *et al* (1991) has suggested that the size of HDL particles may be clinically important. These workers found that smaller particle size was associated with CHD and that particle size was a much stronger predictor of disease than HDL cholesterol.

The dominant components of mature plasma HDL are its proteins, which constitute about 50% of its mass and cover approximately 90% of its surface. They are

responsible for its interaction with enzymes (apoAI is a cofactor for LCAT) and may govern its recognition by receptors on cell membranes. Added to the structural heterogeneity within HDL is the diversity that characterizes its proteins. Of particular interest are the isoforms of apoAI and apoE, which comprise 6 and 3 major variants respectively. The apoAI polymorphism arises largely from post-translational proteolytic cleavage of the protein (Brewer *et al* 1983). The nascent protein first appears within the cell in a pre-pro-form (Law, Gray & Brewer 1983) with a 24 amino acid extension to its amino terminus. A peptide of 18 amino acids is cleaved within the cell to release a pro-protein that is secreted into the circulation. There it undergoes slower proteolytic transformation to the mature protein, with release of the 6 amino acid propeptide. On average, this final step takes about 6 hours. Pro-apoAI is more basic than its mature product and is distinguishable by isoelectric focussing, even although it normally only represents 1-2% of the total apoAI mass. In patients who express the HDL defect known as Tangier Disease, pro-apoAI constitutes a much larger proportion of total apoAI, a finding that initially led to the proposal (Zannis *et al* 1982) that the disease results from defective pro-apoAI maturation. However, kinetic studies have shown (Bojanovski, Gregg & Brewer 1984) that the rates of production and clearance of the pro-protein are normal in this condition. Hypercatabolism of the mature form appears to be responsible for the relative increment in the mass of pro-apoAI in the circulation. ApoAII, the other main HDL protein, is also synthesised in pre-pro-form. However, both pre- and pro-peptides are cleaved intracellularly, and only the mature form is found in the plasma (Gordon *et al* 1983). It is not yet known if there is any link between apoA maturation and the formation of nascent or mature HDL.

Although apoE represents only a minor component of HDL, its presence on some of these particles may have a profound influence on their metabolic behaviour. Particles that contain the protein can be separated from the bulk of HDL by affinity chromatography (Weisgraber & Mahley 1980). They are larger than average and bind with high affinity both to the apoB/E receptor and to the putative apoE receptor on hepatocytes. Thus, the presence of apoE on HDL particles opens up the possibility that they may selectively feed into catabolic pathways controlled by the activities of these two receptors. It has already been noted that apoE exists in several isoforms, the major representatives being E<sub>2</sub>, E<sub>3</sub> and E<sub>4</sub>. These apo E phenotypes influence the amount of apoE associated with HDL, in that individuals with the E<sub>2</sub> phenotype transport more of the protein in that density interval than do those with E<sub>4</sub>. One of two processes may contribute to this phenomenon: either the

E2 containing particles may bind less efficiently to the receptor and therefore accumulate in the plasma (Rall *et al* 1983), or the E2 itself may form covalent disulphide bonds with apoAII, trapping the protein in HDL in a form that is a poor ligand for the receptor (Innerarity *et al* 1978). Indeed, the E-AII complex has been identified by Innerarity and his colleagues (1978) as a physiological mechanism for delaying the catabolism of apoE containing particles.

Early analytical ultracentrifugation studies made it clear that HDL was not monodisperse (DeLalla, Elliott & Gofman 1954) but rather, existed as two or more populations that were originally designated HDL<sub>1</sub>, HDL<sub>2</sub> and HDL<sub>3</sub>. It transpired that 'HDL<sub>1</sub>' actually represented a mixture, the main component of which was a variant of LDL viz. Lp(a). Of the other two, HDL<sub>3</sub> (d. 1.125-1.210 g. mL<sup>-1</sup>) was major and HDL<sub>2</sub> (d. 1.063-1.125 g. mL<sup>-1</sup>) minor in mass terms, but not in clinical significance. Further work in the Donner Laboratory in Berkley, California, resolved HDL into a number of subspecies (HDL<sub>2a</sub>, HDL<sub>2b</sub>, HDL<sub>3a</sub>, HDL<sub>3b</sub>, and HDL<sub>3c</sub>) using gradient gel electrophoresis (Blanche *et al* 1981). The importance of each fraction has been the subject of intensive investigation. The polydispersity that exists in HDL is good evidence for the existence, within the size constraints of this fraction, of a limited number of thermodynamically stable, distinct entities. Moreover, we are now beginning to relate changes in metabolism to alterations in HDL size. In hypertriglyceridaemic individuals, for example, HDL is a small, dense particle that is relatively depleted in cholesteryl ester (Deckelbaum *et al* 1984). As noted above, it seems to be generated by transfer of a proportion of its core cholesteryl ester into the large mass of circulating VLDL in exchange for triglyceride. The action of hepatic lipase on this particle then causes it to shrink as triglyceride and phospholipid are hydrolysed from its core and coat respectively. At the opposite end of the spectrum, patients, who suffer from abetalipoproteinaemia, have extremely low plasma triglyceride levels, and large circulating HDL, which is cholesteryl ester and apoE enriched (Deckelbaum *et al* 1982).

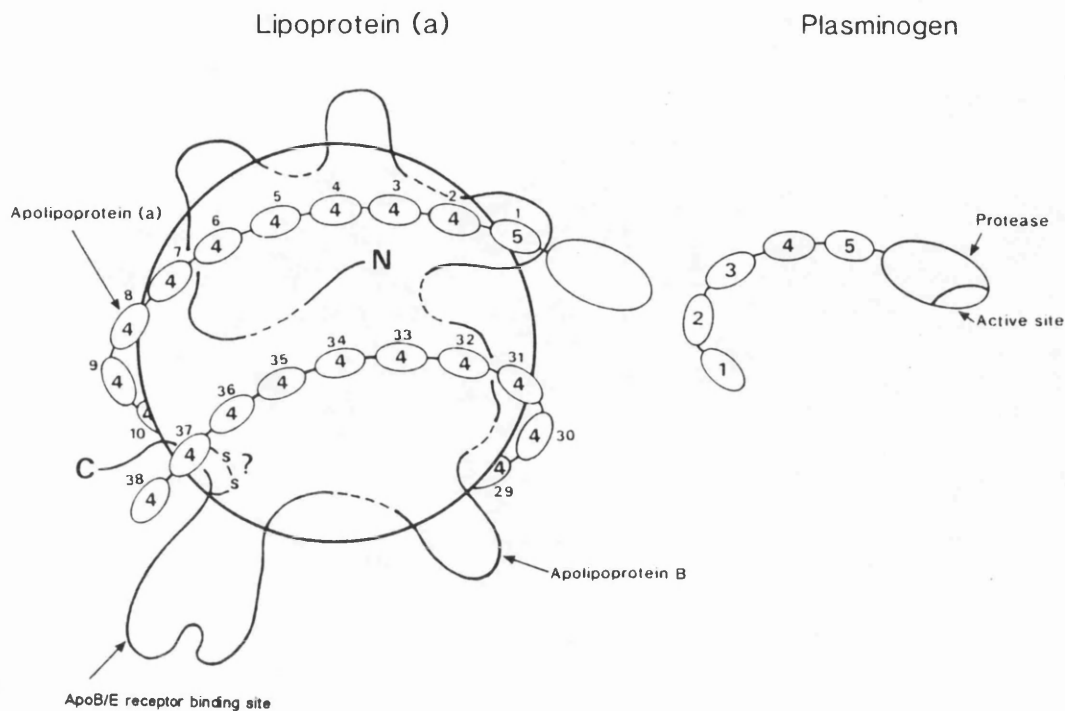
Normal individuals do not remotely approach either of the above extremes, although throughout the day they do exhibit cyclical variations in plasma triglyceride that impact on the distribution of HDL and, indirectly, on coronary heart disease risk. Patsch and his colleagues (1984) have found that individuals who possess an efficient chylomicron clearing mechanism show only minor increments in the flotation rates and negligible changes in the masses of their plasma HDL subfractions following a fatty meal. In such patients, HDL<sub>2</sub> and HDL<sub>3</sub> become enriched in phospholipid, which is gradually lost, probably through the



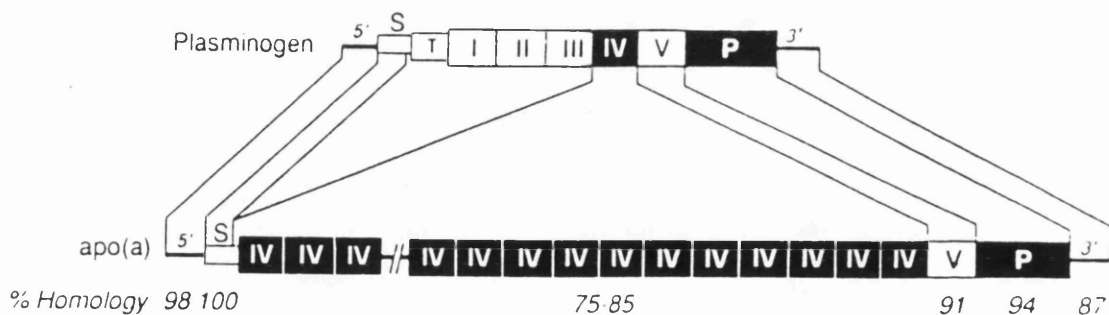
action of hepatic lipase, as the post-prandial lipaemia resolves. HDL probably acts in this situation as a single acceptor of chylomicron coat phospholipid, shed during hydrolysis of the triglyceride-rich particle. At the other end of the normal spectrum exist individuals whose ability to clear chylomicrons is low. They show marked and prolonged lipaemia following a fatty meal. The increased residence time of triglyceride in their plasma facilitates its transfer into HDL in exchange for cholesteryl ester. Phospholipid is also transferred from the chylomicron to HDL during the lipolytic process (Schaefer *et al* 1982) so that these denser particles acquire both coat and core components. This results in an increase in their susceptibility to hydrolysis by hepatic lipase that leads to a shrinkage of the particles and an increase in their density. The magnitude of this change is so great that particles, which initially appeared in the HDL<sub>2</sub> density interval, now isolate as HDL<sub>3</sub> (Patsch *et al* 1984). Such individuals therefore present, even in the fasting state, with a lower HDL<sub>2</sub>/HDL<sub>3</sub> ratio than is found in those whose lipolytic processes are efficient. Reduced HDL levels, and in particular low HDL<sub>2</sub>/HDL<sub>3</sub> ratios have been equated with increased risk of coronary heart disease (Gofman, Young & Tandy 1966). It is possible that this finding is not of primary importance but is merely a reflection of the status of triglyceride-rich particle metabolism. Certainly, Simpson and his colleagues (1990) have found that individuals with angiographically proven coronary heart disease not only have lower HDL<sub>2</sub>/HDL<sub>3</sub> ratios in their plasma but also suffer from a defect in their ability to clear chylomicrons from their bloodstream.

### 1.2.7 Lipoprotein (a)

Lipoprotein (a) [Lp(a)] was first described by Berg (1963), and its structure has since been defined by several groups. Lp(a) is very similar to LDL in its lipid composition and like LDL contains a single copy of apoB-100. In addition it possesses a large glycoprotein called apo(a), which is thought to be linked to the apoB-100 moiety by a single disulphide bridge (figure 3). Apo(a) has been shown to have striking structural homology with plasminogen (MacLean *et al* 1987) (figure 4) and varies in size from approximately 400 kD to more than 800 kD (Utermann *et al* 1987) (figure 4). Human apo(a), like plasminogen, consists of a protease-like sequence, linked to multiple repeat domains held together by three internal disulphide bridges and called kringles. Unlike plasminogen, apo(a) is extensively glycosylated and a single copy of kringle 5 is linked to a variable number of kringle 4 repeats (Gavish, Azrolan & Breslow 1989).



**Figure 3.** Proposed structure of Lipoprotein (a) compared with that of Plasminogen. The numbered domains are the kringles. (adapted from MBewu & Durrington 1990).



**Figure 4.** cDNA structure of Apo (a) in relation to that of plasminogen. (Redrawn from McLean *et al* 1987).

There is much less known about the metabolism of Lp(a) than the other lipoproteins but a summary of present knowledge is provided by MBewu & Durrington (1990). Lp(a) is probably secreted directly into the circulation and is not a product of intravascular delipidation like LDL. Apo(a) is thought to be primarily of hepatic origin but its metabolic fate is unknown.

In an attempt to reveal the determinants of plasma Lp(a) levels, which vary widely between subjects, the genetic basis of the apo(a) size heterogeneity has been uncovered. Genotyping of apo(a) performed by pulsed field gel electrophoresis (Lackner *et al* 1991) has shown that the size of the apo(a) gene correlates directly with the size of the apo(a) protein and inversely with the Lp(a) plasma concentration. Segregation analysis of the apo(a) gene also revealed that in normals the Lp(a) plasma level was largely determined by alleles at the apo(a) gene locus.

A number of studies have demonstrated that Lp(a) is an independent risk factor for coronary heart disease. Most recently, Rosengren *et al* (1990) found, in a prospective study of Swedish middle aged men, that plasma Lp(a) level is an important determinant of risk of myocardial infarction. Wiklund *et al* (1990) have also reported that apo (a) may be useful in the identification of those FH heterozygotes who are particularly at risk of coronary heart disease. But, how does Lp(a) contribute to such risk? Because its structure links it with both lipoprotein and thrombotic risk factors two main lines of research have been pursued in an attempt to answer this question. There is autopsy (Rath *et al* 1989) and biopsy (Cushing *et al* 1989) evidence for the presence of Lp(a) in the intima of the artery wall. Within the intima, Lp(a) is thought to be immobilized by binding extracellularly to glycosaminoglycans (Bihari-Varga *et al* 1988). There is immunological evidence for the presence of Lp(a) in foam cells (Niendorf *et al* 1990), which would suggest that these lipoproteins can be taken up by macrophages and contribute to foam cell conversion. Oxidative modification of Lp(a), which has recently been described by Sattler and coworkers (1991), may render the lipoprotein a suitable substrate for macrophage scavenger receptor-mediated uptake. These workers have also discovered that Lp(a) is much more resistant to *in vitro* oxidation than LDL, perhaps because of its higher N-acetylneuraminic acid content. This relative protection from oxidation may be overcome in part by the prolonged residence of Lp(a) in the intima where it remains bound and trapped, but may also account for the apparently low intracellular levels of Lp(a).

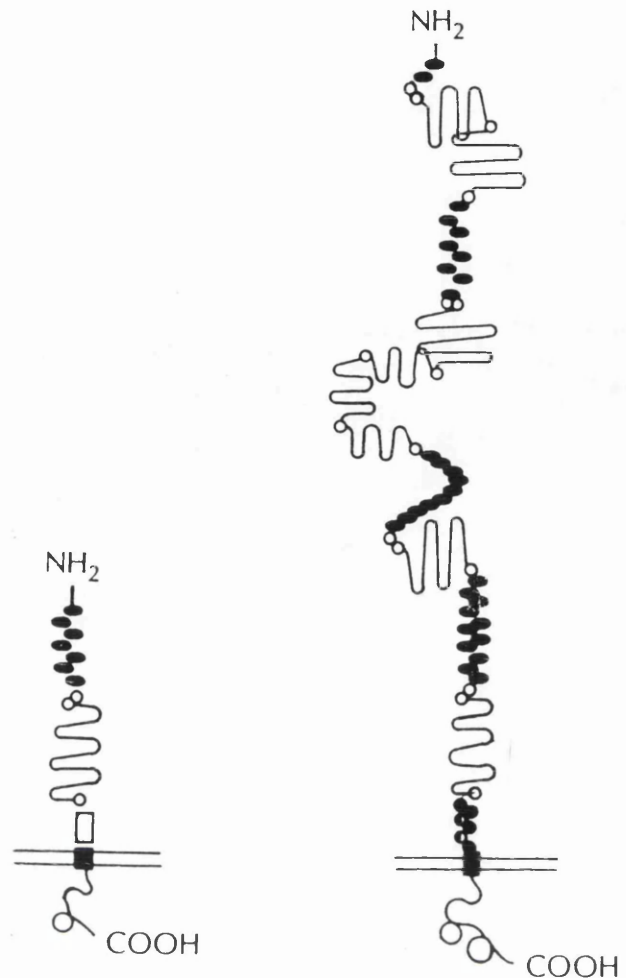
Thrombosis is an important prodromal event in myocardial infarction. Evidence to support this statement comes not only from post mortem evidence of occlusive thrombi in coronary arteries already narrowed by atheroma, but also from the beneficial effect of post-infarction thrombolytic therapy seen in the Second International Study of Infarct Survival (ISIS-2 1988). The other main explanation offered for the atherogenicity of Lp(a) centres on the possible link between this lipoprotein and fibrinolysis and thrombosis. Apo(a) has a high degree of structural homology with plasminogen and has been hailed as the missing link between atherosclerosis and thrombosis. The evidence to date, while supportive of this hypothesis, in no way confirms it. The subject is reviewed by Miles & Plow (1990) who present evidence to suggest that Lp(a) inhibits fibrinolysis by competing for plasminogen binding sites, which in turn leads to an increased likelihood of thrombosis. Fibrinolysis may also be influenced by the effect of Lp(a) on plasminogen activator inhibitor-1 (PAI-1). Etingin *et al* (1991) demonstrated in cultured endothelial cells that Lp(a) regulated the expression of PAI-1, and Edelberg, Reilly & Pizzo (1991) suggest that Lp(a) may regulate fibrinolysis by competing with PAI-1 and plasminogen for fibrinogen or heparin-bound tissue-type plasminogen. The effects of Lp(a) on fibronectin have also been investigated. Ehnholm, Jauhiainen & Metso (1990) have shown that this protein is subject to proteolytic cleavage by apo(a). The importance of each of these findings *in vivo* is as yet unknown but this grey area between the fields of lipoproteins and thrombosis continues to attract extensive research.

Lp(a) is an intriguing particle that has given up few of its secrets despite three decades of research. One of the most fundamental questions, which remains unanswered, is its physiological role. A number of hypotheses have been put forward but one of the most unusual comes from Rath & Pauling (1990) who suggest, based on evolutionary data, that Lp(a) is a surrogate for ascorbic acid.

### 1.2.8 The ApoB/E Receptor and ApoB Metabolism

The discovery and characterization of the apoB/E receptor has become the paradigm of lipoprotein research and was the result of the efforts of Brown & Goldstein (1987). This molecule is a single transmembrane glycoprotein with its 839 amino acids arranged into five functional domains (figure 5). The ligand binding domain is found in the 300 amino acid N-terminal segment, which consists of seven 40 amino acid repeat units, each of which is enriched in aspartate and glutamate residues arranged in a configuration that facilitates electrostatic interaction with

**Figure 5.** Schematic diagrams of the ApoB/E (LDL) Receptor and Low Density Lipoprotein receptor Related Protein (LRP). Ligand binding repeat (●); Epidermal-growth-factor-precursor homology domain [growth factor repeats (○), spacer region (S)]; O-linked sugar (□); Transmembrane (■) (Adapted from Brown *et al* 1991)



complementary arginine and lysine residues found on two apolipoproteins, apoB and apoE (Mahley & Innerarity 1983). The receptor therefore recognises and interacts with particles containing these proteins, but with different affinities since the E protein binds ten times more effectively than the B. In theory then, the whole spectrum within the VLDL-LDL interval, which contains these proteins is substrate for the apoB/E receptor. Early metabolic studies (Bilheimer, Stone & Grundy 1979) focused on LDL since it was the lipoprotein recognised to be markedly increased in familial hypercholesterolaemia (FH). However, as knowledge develops it is becoming clearer that the impact of receptor deficiency is felt along the length of the VLDL delipidation cascade, suggesting that the 'LDL' receptor has a wider role in apoB metabolism than its name suggests.

Tracer kinetic analysis following administration of [<sup>125</sup>I]-labelled LDL to humans has shown that approximately 30-40% of the entire LDL mass in the plasma is catabolised each day. Chemical modification of the tracer with agents such as 1,2 cyclohexanedione (Shepherd *et al* 1979a), glucose (Kesaniemi, Witztum & Steinbrecher 1983), or 2-hydroxylacetaldehyde (Slater *et al* 1984), which interact

with arginine or lysine residues on its apoprotein moiety, reduces its clearance by 50-70%. Since this treatment blocks binding of the lipoprotein to the receptor, clearly receptor activity must make a major contribution to LDL turnover. The role of the receptor falls by half in those subjects with heterozygous FH and is abolished in homozygous individuals (Shepherd *et al* 1979a). In consequence, more LDL is directed into alternative catabolic mechanisms that are mediated via the macrophage scavenger receptors (Matsumoto *et al* 1990).

We know from extensive experiments on cultured cells that the activity of apoB/E receptors is regulated by variation in the intracellular sterol pool. When the requirement for cholesterol is increased, receptor synthesis is stimulated and LDL uptake promoted. Conversely, in times of surfeit, receptor expression is diminished and LDL assimilation suppressed. Extrapolation of these concepts to man has enlightened our understanding of the regulation of LDL metabolism and provided an explanation for the actions of a number of cholesterol lowering drugs. This is discussed further in section 1.6 below.

As indicated earlier, theoretical considerations suggest that we ought to expect the apoB/E receptor to be implicated in the metabolism of VLDL and IDL. In 1982, Soutar, Myant & Thompson discovered that IDL clearance is retarded in FH. This problem was further examined in detail using cumulative flotation ultracentrifugation to follow the flux of apoB from VLDL through IDL to LDL (James *et al* 1989). FH homozygotes accumulate cholesterol-rich remnants within their VLDL density interval because they lack the capacity to clear them normally. IDL catabolism is also perturbed to such an extent that its pool size increases as much as that of LDL, the level of which rises 3.5 fold in the plasma. Absence of the apoB/E receptor, therefore, has a profound impact on apoB metabolism in its entirety. But, what of its role in normolipaemic subjects? This question may be addressed by following the approach outlined earlier that relies on modification of the ligand to affect its receptor binding. Treatment of large, triglyceride-rich VLDL (Packard *et al* 1985) has no effect on its direct clearance from the plasma or its conversion to IDL. However, subsequent catabolism of apoB containing particles is significantly retarded. Additional evidence supporting the view that apoB/E receptors participate in VLDL remnant and IDL clearance comes from clinical observation of patients with dysbetalipoproteinaemia. This condition is accompanied by high circulating levels of both these lipoprotein fractions due to their inability to bind efficiently to hepatic lipoprotein receptors. Up-regulation of the apoB/E receptor with an HMG CoA reductase inhibitor lowers the concentration

of VLDL remnants and IDL in the plasma of these patients (Vega, East & Grundy 1988).

Familial hypercholesterolaemia (FH), the autosomal codominant trait that occurs in its heterozygous form in 1 in 500 of the population is associated with high levels of LDL cholesterol in the plasma and premature coronary heart disease and is known to arise because of a defect in the apoB/E receptor. Since its discovery more than 150 different mutations of the apoB/E receptor have been described (Hobbs *et al* 1990) that are responsible for FH. The many deletions, insertions and base pair substitutions within the apoB/E receptor gene can have profound effects on LDL catabolism. Recent observations suggest that similar and equally deleterious effects may result from defects in its primary ligand, apoB-100. This is most commonly seen in a condition called familial defective apoB-100, which is due to a mutation at a CG dinucleotide in residue 3500 of the apoB gene and is present again at a level of approximately 1 in 500 of the population (Innerarity *et al* 1990). Such changes in apoB sequence appear to be of real clinical significance and further mutations are currently being sought.

### 1.2.9 The HDL Receptor and Reverse Cholesterol Transport

From the discovery that plasma HDL levels are associated with protection from coronary heart disease (reviewed by Gordon *et al* 1989) came the concept that this lipoprotein acts as a vehicle to transport sterol from peripheral tissues to the liver. The necessity for such a mechanism is unquestionable since the perhydrocyclopentanophenanthrene nucleus of cholesterol cannot be catabolized to any significant extent in animals and must therefore be excreted intact via its major organ of elimination, the liver. HDL has the capacity to acquire cholesterol from cells *in vitro* as an initial step in the 'reverse cholesterol transport pathway' advocated by Glomset (1968).

The precise mechanism responsible for uptake is, however, currently disputed. It is generally accepted that the free sterol, rather than its ester, participates in the transfer process. Whether this occurs during cell-lipoprotein collisions, as a result of passive diffusion, or through capture and release of HDL by cells, is not clear. The binding process would require the specificity inherent in a receptor protein, and there have been several publications claiming the detection of proteins that appear to meet this requirement. If such a mechanism exists, the binding site should be saturable and subject to regulation, on the basis, for example, of cell cholesterol

content. Biesbroecke and his colleagues (1983) have evidence to suggest that the interaction of HDL with fibroblasts displays these characteristics and promotes cholesterol release from cells. Binding can be inhibited by alteration of the tyrosyl residues of the HDL protein with tetranitromethane (Tabas & Tall 1984; Chacko 1985) but is unaffected by arginine and lysine modification. The binding process seems specific for HDL proteins. LDL fails to compete, while liposomes containing apoAI, the major protein in HDL, bind with high affinity. Recently, Tozuka & Fidge (1989) have confirmed the presence of cell surface proteins that bind both HDL proteins, AI and AII. Furthermore, these workers report that HDL<sub>3</sub> is the principal ligand for these receptors and that LDL was unable to compete. The removal of cholesterol from peripheral cells may also occur via an elegant internalization process involving the whole HDL particle. Postulated by Alam *et al* (1989), this process results in the intracellular remodelling of HDL, increasing its size and apoE content, after which the particle emerges from the cell by a process of 'retroendocytosis'. Against the receptor concept, however, is the evidence that limited proteolysis of either the lipoprotein or the cell membrane surface fails to inhibit binding. Nevertheless, data from two sources suggest that the binding process is regulated by the free cholesterol content of the cell and may be modulated by mitogens such as platelet derived growth factor (Oppenheimer, Oram & Bierman 1987), which was shown to decrease both HDL binding and cholesterol efflux.

A fascinating development in this field is offered by Milda, Fielding & Fielding (1990) who give supportive evidence for an hypothesis that the functional heterogeneity of HDL encompasses the ability of this lipoprotein to process cholesterol from peripheral cells and other plasma lipoproteins independently. This further refinement in our understanding of HDL metabolism only serves to remind us of how much we have still to learn.

#### 1.2.10 The ApoE Receptor and Chylomicron Metabolism

As noted above, dietary lipids are packaged within the enterocytes of the small intestine into large triglyceride-rich chylomicron particles that contain approximately 1-2% protein. The latter consists primarily of apoAI, apoAII, apoAIV and apoB-48. This shortened B protein lacks the apoB/E receptor binding site, and the chylomicron must acquire an alternative receptor-binding ligand before it can be taken up by the liver (Chen *et al* 1987). This occurs soon after the particle enters the circulation as a result of transfer of apoE, principally from HDL. Lipolysis of the triglyceride core of the chylomicron in the capillary beds of skeletal muscle and



adipose tissue generates a relatively cholesterol enriched remnant, which is purported to be atherogenic since it is able to produce cholesteryl ester deposition in macrophages. Teleologically, there is biological benefit in clearing these remnants as quickly as possible from the bloodstream. This function is performed by the liver, which has a highly efficient uptake mechanism capable of clearing the blood of these remnants in a single pass. The apoB/E receptor could in theory participate in this process but is unlikely to do so since chylomicron remnant metabolism is not compromised in homozygous FH subjects (Rubinstein *et al* 1990). The most promising putative chylomicron remnant receptor (also called the apoE receptor) is the LDL receptor-related protein (LRP) (Herz *et al* 1988). LRP is a cell surface protein that is much larger than the apoB/E receptor but is structurally homologous, comprising the elements of four apoB/E receptors (figure 5). LRP has been shown to be an apoE binding protein (Beisiegel *et al* 1989) and to mediate the uptake of cholesteryl esters derived from apoE enriched lipoproteins *in vitro* (Kowal *et al* 1989). One of the most intriguing aspects of this newly defined protein is the observation that it acts as a receptor for the plasma protein  $\alpha_2$ -macroglobulin (Strickland *et al* 1990). If LRP does have two apparently unrelated ligands, apoE and  $\alpha_2$ -macroglobulin we are faced with some difficult problems. What will the primary function of this protein be *in vivo*, how may its expression be manipulated, and how could such a double receptor protein evolve? Some attempt to shed light on these important questions is made by Brown *et al* (1991) but they conclude that much more work has to be done before we will be in a position to accept the LRP as the definitive chylomicron remnant receptor that has been sought for so long.

### 1.2.11 Modified Lipoproteins and the Scavenger Receptors

Detailed electron microscopic studies have shown that in cholesterol fed non-human primates the earliest prodromal sign of a developing atherosclerotic plaque is focal infiltration of the subendothelial space of the arterial wall by cells of the monocyte/macrophage series (Fagiotto, Ross & Harker 1984). When examined in culture, these cells are able to assimilate and deposit cholesteryl esters in intracellular lipid inclusions (Brown & Goldstein 1983). The source of this sterol has been the topic of a large body of research. Plasma LDL, the most abundant sterol transporter, does not generate these deposits. However, modified LDL particles extracted from the aorta (Hoff & Morton 1987) have this ability. They differ from the normal lipoprotein by being more electronegative, like the artificially produced acetyl LDL, which is avidly assimilated and deposited in macrophages (Goldstein *et al* 1979). Indeed, these cells exhibit on their membranes proteins that

facilitate the rapid unregulated uptake of acetyl LDL to the extent of generating foam cells reminiscent of those found in the atherosclerotic lesion. Because a wide variety of negatively charged compounds can compete with acetyl-LDL for these receptors they have become known as scavenger receptors (Brown *et al* 1980). Clearly, the physiological ligand for these receptors is not acetyl LDL, nor are they present to facilitate the production of foam cells. Steinberg *et al* (1989) have found that charge modified lipoproteins, in the form of oxidized LDL, compete with acetyl-LDL and cause foam cell conversion. They concluded that modified lipoproteins may be the *in vivo* ligand for these scavenger receptors. A number of observations support the hypothesis that oxidized LDL is important in the development of atheromatous plaques. Firstly, there is immunological evidence for the presence of oxidized LDL in atheromatous lesions and even human fatty streaks (Haberland, Fong & Cheng 1988; Yla-Herttuala *et al* 1989, 1991). Secondly, LDL isolated from atherosclerotic plaques (Goldstein *et al* 1981; Yla-Herttuala *et al* 1989) is electronegative and produces foam cell transformation *in vitro*. And, thirdly, Carew, Schwenke & Steinberg (1987) have attributed the protective action of the drug probucol to its ability to scavenge free radicals and limit the rate of LDL oxidation, rather than to its lipid lowering properties. When the drug was administered to Watanabe rabbits, LDL uptake into atherosclerotic lesions fell by 65%, while a comparative group given alternative lipid lowering drugs showed no improvement.

The mechanism for the modification of LDL *in vivo* is speculative. Evidence from tissue culture work (Steinbrecher *et al* 1984; Henricksen, Mahoney & Steinberg 1987) suggests that endothelial cells or even macrophages themselves, through their ability to generate superoxide radicals, may initiate oxidative damage to the lipoprotein's lipid and protein moieties. This could obviously occur exclusively within the artery wall, accounting for the inability to detect the damaged or modified lipoproteins in the plasma (Frei *et al* 1988).

There is evidence to suggest that multiple scavenger receptors must exist on the macrophage membrane (Sparrow, Parthasarathy & Steinberg 1987) and it is now known that these cells express at least two types of scavenger receptor protein that have been isolated and cloned (Kodama *et al* 1990, Rohrer *et al* 1990). Both human type I and type II scavenger receptors have been localized on foam cells in atheromatous plaques (Matsumoto *et al* 1990). The presence of macrophage scavenger receptor mRNA in atherosclerotic lesions with little associated apoB/E receptor mRNA (Yla-Herttuala *et al* 1991) further points to a key role for these

scavenger receptors in the uptake of cholesterol and conversion of macrophages to foam cells.

The effect of oxidized lipoproteins on vascular tone is another important area (reviewed by Henry & Bucay 1991) where lipoproteins have been shown to have effects on the cardiovascular system in addition to their long-recognized role in atherogenesis. Oxidized LDL has been found to impair arterial relaxation *in vitro* in response to agents that act by stimulating the release of endothelium-dependent relaxing factor (EDRF) from endothelial cells. Clinical reports (Vita *et al* 1990; Zeiher *et al* 1991) have further raised interest in this area by indicating that hypercholesterolaemia may be associated with a decreased responsiveness to endothelium dependent vasodilators (e.g. acetylcholine). This defect, it is suggested, may seriously limit vasodilatory reserve and contribute in part to myocardial ischaemia in such patients. Such a vasomotor syndrome resulting from hypercholesterolaemia resembles the *in vitro* observations that have been explained by the presence of oxidized LDL. This work is still at an early stage but clearly if confirmed will have profound implications for clinical management.

Oxidative modification of LDL has also been postulated as one of the very first steps in the development of the fatty streak. Mild oxidation of LDL particles, resulting in so-called, minimally-modified LDL (MM-LDL), gives rise to a lipoprotein that is not chemotactic in itself but that stimulates the release of monocyte chemotactic protein-1 (Berliner *et al* 1990; Cushing *et al* 1990). Moreover, MM-LDL has also been shown to promote endothelial-monocyte binding, an important step in early atherosclerotic plaque formation. A further putative link between lipoproteins and the thrombotic system has been put forward by Drake *et al* (1991) who showed that MM-LDL when cultured with human endothelial cells induces the expression of tissue factor (tissue thromboplastin), which may be involved in platelet adhesion.

### 1.3 Methods for Studying Lipoprotein Metabolism

While *in vivo* experiments in man are the studies of choice to provide the most biologically relevant data on human lipoprotein metabolism, a number of practical and ethical constraints have necessitated the use of *in vitro* systems such as cell culture and isolated perfused organs. Although the studies of apolipoprotein B metabolism described in this thesis have involved human *in vivo* techniques exclusively it is pertinent in this general discussion to include a short section on *in vitro* methodology.

As outlined in section 1.2 normal lipoprotein metabolism depends on the coordinated actions of a number of enzymes, lipid transfer proteins and specific cell surface receptors. *In vivo* experiments can provide information on the integrated processes of lipoprotein interconversions, but in order to study individual components of this system, tightly controlled experimental models must be designed. However, if the *in vitro* system under study is to have any physiological relevance a number of basic rules, as set forth by Eisenberg (1986) should be followed:

- a) Undamaged lipoproteins should be used in preference to adulterated preparations, lipid emulsions, or artificial lipid:protein complexes.
- b) Lipoproteins under study should be exposed to multiple reactants, as they are *in vivo*.
- c) Continuous metabolic cycling must be permitted in the *in vitro* system or the interruption of such cycles should be recognised.

These rules are attempts to recreate the physiological state in the test tube, and serve as reminders that while we may simplify a system in order to make it accessible to study, we may, in so doing, render it fundamentally different from the original and therefore biologically irrelevant.

It is important, however, to realise that *in vivo* and *in vitro* studies are complementary; many aspects of lipoprotein metabolism having only been elucidated by the application of both approaches. Greater confidence is naturally placed in the results if several entirely independent techniques have arrived at consistent answers.

### 1.3.1 Cell Studies.

Studies involving cultured human skin fibroblasts have contributed a great deal to our understanding of lipoprotein metabolism. The discovery and characterization of the apoB/E receptor by Goldstein and Brown was by means of a series of experiments that used fibroblasts from patients with homozygous and heterozygous FH (Brown, Faust & Goldstein 1975). Cell culture studies using in addition to fibroblasts, hepatoma cell lines, e.g. Hep G2 cells, cultured macrophages and hepatocytes, are the most widely used methods in the study of lipoprotein metabolism. They have undoubtedly allowed many of the cellular and intracellular events of lipoprotein synthesis and catabolism to be elaborated, but the results

obtained from such studies should always be subject to validation by *in vivo* experiments either in animals or, preferably, man.

### 1.3.2 Perfused Organ Studies.

The study of hepatic lipoprotein metabolism has been made possible by measuring the output of isolated, perfused livers (Marsh 1986). Livers of animals as large as pigs have been successfully perfused in studies of lipoprotein metabolism (Nakaya *et al* 1977) but the most convenient and therefore most widely used animal liver is that of the rat. Perfusion experiments may be carried out *in situ* (Hamilton *et al* 1974) or in an extracorporeal apparatus (Felker *et al* 1977). Re-circulation of the perfusate provides an isolated organ system that is close to the physiological state, but does not allow for measurement of hepatic lipoprotein synthesis due to hepatic catabolism of secreted products. Alternatively, single pass perfusion may be used in isolated organs. This technique has the advantage that it prevents lipoproteins or enzymes released by the liver from being continuously exposed to hepatic degradation, but also has the disadvantage that huge volumes of perfusate are generated. Other perfused organs have included the rat heart (Chajek & Eisenberg 1978) to examine the effects of endothelial bound lipase on triglyceride rich lipoproteins, and the intestine (Windmueller, Herbert & Levy 1973) to investigate intestinal apolipoprotein synthesis.

### 1.3.3 In Vivo Study of Lipoprotein Metabolism Using Radioisotopes.

Early studies of plasma cholesterol and triglyceride kinetics were based on the use of labelled precursors ( $[^3\text{H}]$ glycerol, Farquhar *et al* 1965,  $[^{14}\text{C}]$ fatty acids, Baker & Schotz 1964) that were incorporated into the plasma lipid fractions. Measurement of their clearance from the bloodstream gave preliminary crude information on whole body turnover rates. However, the limitations of this approach became apparent when it was realised that plasma lipids were associated with specific proteins that are capable of exchange between the different lipoprotein species. Consequently, most of the recent metabolic studies have used an exogenous labelling procedure in which the lipoprotein or apolipoprotein under study is isolated and trace-labelled, usually by iodination. The tracer is then introduced back into the subject and frequent timed blood samples are drawn to follow its catabolism. The plasma disappearance rate of radioactivity from the protein associated with a specific lipoprotein fraction is used as an estimate of the fractional catabolic rate (FCR) of the protein; this is expressed in 'pools catabolized per day'.

Determination of the plasma pool itself (in mg) permits the calculation of the absolute catabolic rate (ACR) of the protein (in mg per day), which is usually expressed per kilogram body weight. In 'steady state', this is numerically equal to the apolipoprotein synthetic rate. Building on early work on the metabolism of free fatty acids and triglyceride turnover, this method was first applied to lipoproteins in the early investigations of Gitlin *et al* (1958), but most of the present day work in lipoprotein kinetics is derived from the studies of Langer, Strober & Levy (1972). They were the first to isolate a defined lipoprotein class (LDL) and determine the synthetic and catabolic rates of its protein component (apoB).

The validity of exogenous labelling techniques is dependent on the following assumptions:

- a) The labelling process does not alter the structure or function of the lipoprotein under study, i.e., tracer and tracee are metabolically equivalent.
- b) The tracer is structurally and metabolically homogeneous.
- c) Catabolism occurs in or close to the plasma compartment or in a pool that is in rapid equilibrium with the plasma.
- d) The concentration (pool) of the tracee plasma lipoprotein does not change during the period of study, i.e., there is no long term trend or periodic rhythm.

The first assumption is very important and the attempts to validate this must be reviewed. The problem of tracer/tracee equivalence was first examined by Langer and his colleagues who showed that iodinated lipoproteins had the same electrophoretic, immunological, and flotation characteristics of unlabelled autologous particles (Langer *et al* 1972). Similarly, several other groups (Eisenberg *et al* 1973; Nestel *et al* 1983; Sigurdsson, Nicoll & Lewis 1975; Kissebah *et al* 1982) have examined this very important issue and concluded that iodination of a lipoprotein does not affect its properties on cellulose acetate electrophoresis, immunoelectrophoresis, gel chromatography or heparin affinity chromatography.

Isolation and radioiodination of a lipoprotein may not render it significantly different from other isolated lipoproteins but this does not mean that it is metabolically indistinguishable from circulating lipoproteins that have never been exposed to *ex vivo* processes. Some experimental data (Berman *et al* 1978) suggest that a portion of such radioiodinated lipoproteins is frequently cleared by a rapid catabolic pathway, after reinjection, assumed to be a consequence of tracer damage.

Comparisons, however, between VLDL apoB and LDL apoB kinetic parameters in hypertriglyceridaemic individuals derived from exogenous radiolabelling (Kissebah *et al* 1976; Sigurdsson, Nicoll & Lewis 1976), and endogenous labelling with labelled amino acids (Fisher *et al* 1980) are very similar.

The methods used to analyse the protein radioactivity data may significantly affect the final interpretation of the results. A number of mathematical procedures have been used (Berman, Grundy & Howard 1982), including curve peeling, deconvolution analysis, interactive simulation, and multicompartmental modelling. The last, originally applied to lipoprotein systems by Berman and his colleagues (1978), is the most versatile and can be used as a tool to test quantitative hypotheses about the system under study. The use of amino acid precursors to follow apolipoprotein kinetics has not been a widely applied technique because of reluctance to give the large doses of [ $^{14}\text{C}$ ] needed to obtain meaningful results. Fisher and coworkers (1980) have provided some data on the incorporation of [ $^{14}\text{C}$ ]leucine into apoB containing lipoproteins while Eaton & Kipnis (1972) used [ $^{75}\text{Se}$ ]selenomethionine. Synthetic rates can be measured directly with labelled precursors if a good estimate of the immediate (usually hepatic) precursor amino acid pool can be obtained. Recycling of the tracer species usually limits the application of these methods to rapidly turning over species like VLDL. Complex computer modelling, which accounts for re-utilisation of labelled amino acids is required if species with prolonged half-lives, e.g. LDL and HDL, are studied.

#### **1.3.4 In Vivo Study of Lipoprotein Metabolism Using Stable Isotopes.**

The potential of endogenous labelling has been further investigated more recently using stable isotope labelled precursors. The use of stable isotopes in biological research is hardly new since the first isotopic tracer studies used the stable isotope of hydrogen to study fat metabolism in mice (Schoenheimer & Rittenberg 1935). Their application to lipoprotein kinetic research is, however, more recent and has been facilitated by recent advances in mass spectrometry technology. The kinetic work performed thus far has produced comparable results with exogenous radiolabelling studies (Cryer *et al* 1986). The general problems inherent in endogenous labelling are also applicable to stable isotope technology (Wolf 1984), but an important advantage in using stable isotopes is that of increased patient safety and acceptability. The analysis of stable isotope data cannot, however, be viewed as directly analogous to that of radioisotope data (Cobelli *et al* 1987). Stable

isotope precursors used have been [ $^{15}\text{N}$ ]-glycine (Cryer *et al* 1986, Schauder *et al* 1989),  $\text{D}_3$ -leucine (Cohn *et al* 1988) and [ $^{13}\text{C}$ ]-phenylalanine (Shaefer *et al* 1988). Early results with this technology are encouraging and provide data that closely mirror those obtained with radio-iodinated tracers. Such studies are of course still hampered by the problem of re-cycling that is seen with radioactive endogenous precursors. If further validated the use of such stable isotope protocols may be extended for the first time to the study of lipoprotein kinetics in children and in pregnancy, as well as multiple studies in the same patient. In spite of these advantages, it is difficult to envisage how this technology would ever completely replace the use of exogenously labelled tracers, and the information it provides should be considered complementary to radioisotope studies.

## 1.4 Methods of Analysis

All kinetic experiments in complex systems such as the human body present two problems. The first is how to collect the data, and the second is how to interpret it. The latter is often no less daunting than the former. Section 1.3 described methods for gathering information on lipoprotein interconversion and catabolic rates; the discussion that follows outlines various mathematical analyses that may be applied to the raw data to extract physiologically meaningful parameters. It should be remembered that all such analyses are approximations to reality, and all involve assumptions that are often little more than intelligent guesses as to what is happening in the body.

### 1.4.1 Urinary Excretion as a Measure of Catabolism

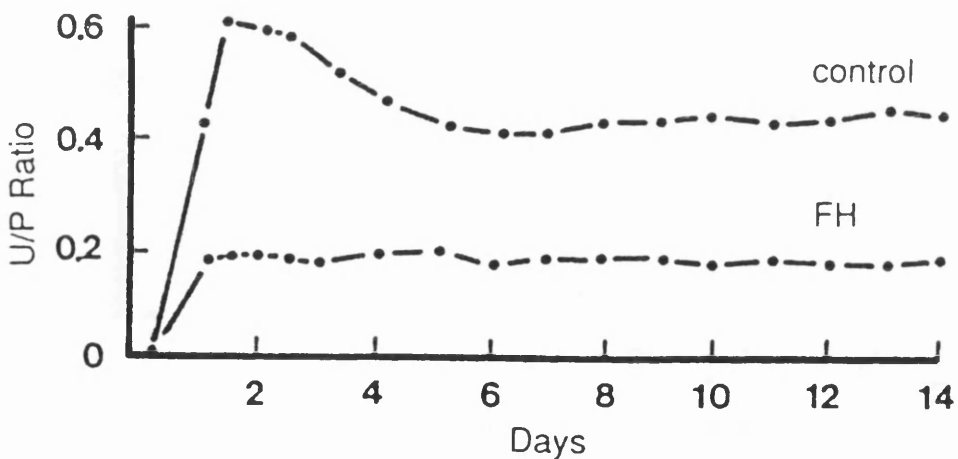
If the site of irreversible loss of trace-labelled material is restricted to the plasma or a compartment in rapid equilibrium with it then the ratio of urinary radioactivity to that in plasma is a measure of the fractional catabolic rate (FCR) of the lipoprotein. This urine/plasma ratio (U/P ratio) is valid if it can be demonstrated that:

- a) urine is the only site of loss
- b) the level of small-molecular weight degradation products in plasma is negligible
- c) the tracer is homogeneous
- d) urine collections are complete over each 24 h period

Early studies by Berson & Yalow (1957) established the use of these urinary radioactivity measurements as a means of assessing the catabolism of iodinated proteins, and the lipoproteins, LDL and HDL, can usually be analysed by this



method. Normally the total urine radioactivity in a 24 h period is measured and related to the mean plasma radioactivity during the same period. With radioiodinated tyrosine there is normally a 0.5 day delay between its appearance in the plasma and its excretion in the urine. Thus a better measure of the mean plasma c.p.m. may be taken as the radioactivity at the start of the 24 h period. It is usual to collect urine continuously over the 14 d of an LDL or HDL turnover and examine the daily U/P ratio (figure 6). Deviation from a steady value may be due to tracer damage (especially high peak values in the first two days) or more likely tracer heterogeneity. The latter is readily seen in most LDL turnovers in normal subjects but not in those with hypercholesterolaemia (Packard *et al* 1976). The U/P ratio is a valuable parameter and can be viewed as a daily survey of the catabolic potential of the tracer. A mean of all 14 U/P values may be used as a measure of the FCR of LDL and it bears a close resemblance to the value obtained by analysis of plasma data. However, the suggestion (Turner *et al* 1984) that a single U/P ratio estimate at day 7 of an LDL turnover may be sufficient to determine the LDL-FCR precisely is not valid (Vega & Grundy 1989a).



**Figure 6.** Urine/plasma radioactivity ratios determined throughout the 14 day period following injection of [ $^{125}$ I] apo-LDL into a control subject and one with heterozygous FH. The inconstancy of the ratio in the control subject probably reflects metabolic heterogeneity within the LDL spectrum. This phenomenon is less apparent in the FH heterozygote.

Urine data are a valuable source of information in LDL and HDL turnovers particularly if a multicompartmental approach is used in analysis as described below. It is an important marker of catabolism and should be included, where possible in all protocols.

### 1.4.2 Determination of Fractional Catabolic Rate from Plasma Data

When daily plasma radioactivities are plotted on semi-logarithmic graph paper, most lipoproteins and apolipoproteins display bi-exponential kinetics, i.e., the decay curve can be described by equation 1, where  $C_1$  and  $C_2$  are the intercepts on the Y-axis, and  $b_1$  and  $b_2$  are the slopes of the two exponentials (figure 7).

Equation 1	$Y=C_1e^{-b_1t} + C_2e^{-b_2t}$
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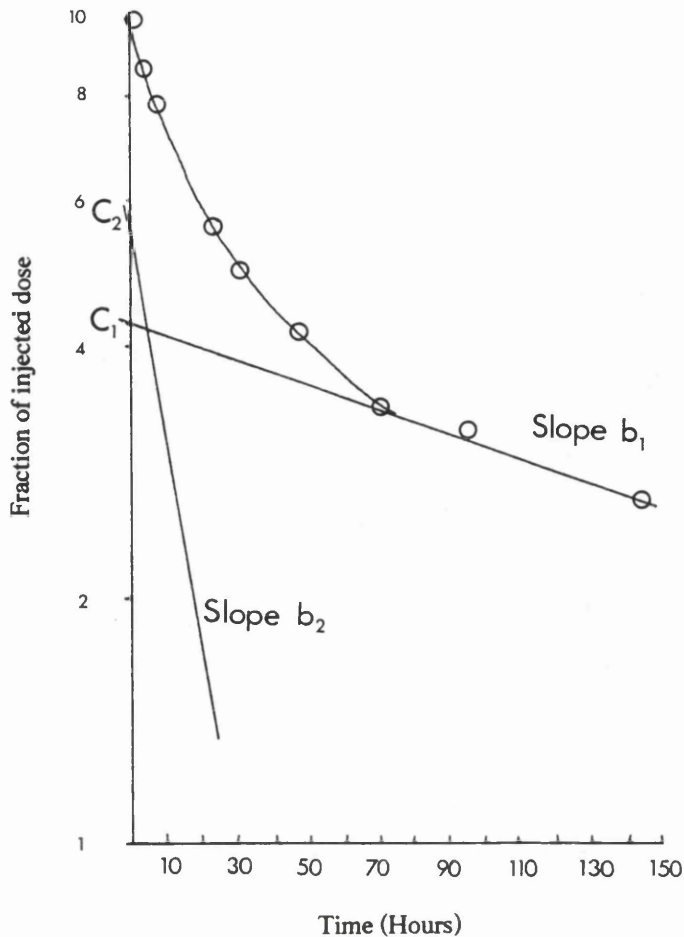
If the tracer is homogeneous and catabolized solely from the plasma compartment then a number of kinetic parameters may be determined by the mathematical approach of Matthews (1957), viz. the fractional catabolic rate (equation 2), the percentage of intravascular tracer (equation 3), and the capillary transfer rate (equation 4)

Equation 2	$FCR= \frac{1}{(C_1/b_1+C_2/b_2)}$
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Equation 3	$\% \text{ intravascular}= \frac{(C_1/b_1+C_2/b_2)^2}{[C_1/(b_1)^2+C_2/(b_2)^2]}$
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Equation 4	$CTR= \frac{C_1C_2(b_2-b_1)^2}{(C_1b_2+C_2b_1)}$
------------	--

Practically, the plasma data are plotted to determine the start of the second, slow exponential. Simple regression can then be used to provide a line of best fit to this exponential, yielding  $b_1$  and  $C_1$ . The first exponential is then extrapolated to the Y-axis and these projected values are subtracted from the plasma curve at suitable intervals (e.g. 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0 days in an LDL turnover) to calculate the second exponential. Linear regression is used again to derive  $b_2$  and  $C_2$ . If the second exponential is curvilinear then further ‘curve-stripping’ is needed and extra terms must be added to the kinetic equations but usually two exponentials are sufficient for analysis of LDL and HDL decay curves.



**Figure 7.** Analysis of biexponential plasma radioactivity curve by resolving into two monoexponentials defined by the parameters  $b_1$  and  $b_2$  (slopes) and  $C_1$  and  $C_2$  (intercepts).

Determination of the plasma FCR by either the Matthews approach or the U/P ratio permits an absolute catabolic rate (ACR) to be calculated i.e., FCR x mass of apolipoprotein in the circulation. If it can be demonstrated that the subject is in steady state then the ACR is equal to the synthetic rate of the apolipoprotein. Pragmatically we accept steady state if:

- the subject's weight has not altered over the study period,
- there is no trend (i.e., gradient of  $>10\%$ ) in the plasma concentration of the lipoprotein under study,
- the coefficient of variation of serial estimations is less than  $10\%$

### 1.4.3 Analysis of Precursor-Product Specific Activity Curves

Studies of lipoprotein interconversions such as the VLDL to LDL delipidation cascade have been analysed using a variety of techniques. A popular approach has been to calculate the specific activities (c.p.m. per mg apoB) of the precursor lipoproteins (VLDL) and the products (LDL or IDL and LDL). The relationship between these curves reveals something of the metabolic links between the lipoprotein species.

Zilversmit (1960) has shown that if a labelled precursor, A, is the sole source of the product, B, then the decay curve for the specific activity of A will cut the product curve at the maximum (peak) of B. If the specific activity of B is less than the specific activity of A at the time of the peak then other 'cold' input is contributing to the mass of pool B. This simple analysis was used in early studies to establish the link between apoB in VLDL and LDL. Furthermore, the areas under the precursor and product curves may be used to derive quantitative measures of the VLDL-LDL conversion rate (Soutar *et al* 1982).

A more sophisticated technique for mathematical analysis of specific activity data has been used by Sigurdsson (1982). Deconvolution or impulse analysis represents an extension of indicator dilution theory. It relies on:

- a) obtaining disappearance curves for [ $^{125}\text{I}$ ]VLDL apoB and [ $^{131}\text{I}$ ]LDL apoB,
- b) measuring an appearance curve for [ $^{125}\text{I}$ ] apoB in LDL, and
- c) unequivocal extrapolation of the precursor curves to infinity (i.e., to 'zero').

The above techniques, which depend on the relationships between curves have been termed 'model independent' and to a certain extent this is valid since they make few assumptions about the system under investigation. The term, however, is misleading in that fundamental preconceptions such as precursor and product homogeneity are inherent in the functions used for analysis. The approach is also limited in the amount of information it can derive from a given data set.

### 1.4.4 Multicompartmental Modelling

Of the various methodologies associated with its study, it is the use of compartmental modelling that has resulted in the formation of a uniform theory of lipoprotein metabolism (Zech, Boston & Foster 1986). Data fitting using

compartmental or mechanistic models is a powerful, general approach to the study of lipoprotein kinetics. It has been used successfully in the analysis of both lipid and apolipoprotein turnovers and has been of great importance to the elucidation of the metabolism of apoB. Building on the original lipoprotein turnovers performed by Gitlin and coworkers (1958) who observed the transfer of tracer from the Sf 10-100 interval to the Sf 3-9 (i.e., from VLDL to LDL), an apo B 'delipidation chain' was first included in a multicompartmental model by Phair and his colleagues in 1975. This work was extended at the NIH and a new mechanistic model of apoB metabolism was proposed in 1978 that incorporated a slowly turning over VLDL compartment (Berman *et al* 1978). Since that time several groups have proposed alterations of the multicompartmental model that is used as an hypothesis of apo B metabolism. This evolution of a model, based on new experimental observations, is an essential part of its character, however, as models become increasingly complex in order to explain metabolic reality they become increasingly abstract and inaccessible to the user. One of the main disadvantages in using multicompartmental analysis is that these detailed, structured models have to be specified based on the physiological processes under investigation, but often they contain features necessary for the solution that cannot be attributed to a known metabolic step. In any modelling process it is vital to remember that the model in use is simply a model, i.e., a mathematical representation of reality. The solutions we obtain are mathematical approximations to the metabolic reality based on the constraints of the model.

The most powerful tool that has been used in this area of lipoprotein metabolism is the Simulation, Analysis And Modeling (SAAM) computer program generated by Berman & Weiss (1974). This is a package originally devised to operate in a batch mode but is now available in an interactive version, CONSAM (Boston, Greif & Berman 1982). This form of the program has, as its acronym suggests, a conversational potential for interrogation, display, modification of the model and the solutions, and for setting up and executing various modelling tasks. These modelling programs, given an observed data set and initial estimates, will alter the parameters in an iterative manner until they reach a minimum sum of squares for the residual differences between observed and calculated data.

Multicompartmental modelling can be used for parameter estimation in complex metabolic pathways and is limited mainly by the quality and quantity of information contained in the data set. It also allows quantitative hypotheses to be generated and tested.

## 1.5 Concepts of Hypolipidaemic Therapy.

With the decision to measure an individual's lipoprotein profile must go the willingness to offer rational management if a dyslipidaemia is detected. The form of that management may range from simple verbal advice to lifelong multiple drug therapy or even surgery. Clearly, such decisions must not be taken lightly and must be based on the best knowledge available. In addition, the treatment of dyslipidaemia must not be separated from the management of the other main correctable risk factors, smoking and hypertension. In recognition of this fact some hospital lipid or lipoprotein clinics have changed their names in recent years to Risk Factor Clinics (Evans, Taylor & Taylor 1990).

### 1.5.1 Criteria for Therapy

The decision of who should receive management of dyslipidaemia has been clarified to some extent by a series of major reports. The guidelines issued by the British Hyperlipidaemia Association (Shepherd *et al* 1987), the Study Group, European Atherosclerosis Society (1988), and the National Cholesterol Education Program (1988) provide a consensus on the management of patients with elevated cholesterol levels. It is clear from an inspection of these reports that a number of issues have not been addressed, viz. how age and sex influences the criteria for management. There is still, therefore, some controversy over these issues and they will only be resolved by further clinical research involving large primary and secondary CHD prevention studies.

### 1.5.2 Therapeutic Options. Non-pharmacological.

Once hyperlipidaemia has been confirmed by repeated testing and the main secondary forms have been excluded by clinical and biochemical monitoring of endocrine and renal function a number of therapeutic options exist. Published guidelines for the management of patients with hyperlipidaemia concur that diet is of prime importance and they advocate the first line use of a general lipid lowering diet or 'step-one diet' (Consensus Conference 1985; Shepherd *et al* 1987; National Cholesterol Education Program 1988; Study Group, European Atherosclerosis Society 1988). This step-one diet involves: reducing calorie intake to achieve and maintain desirable body weight; reducing total fat intake to less than 30% of total calorie intake; increasing the ratio of polyunsaturated to saturated fat intake to 1:1 and taking 10-15% of total calories as monounsaturated fat; and reducing dietary cholesterol intake to less than 300 mg. d<sup>-1</sup>. This advice, and variations on it, form the cornerstone of the initial management of hyperlipidaemia. While the response to

this step one diet has been criticised by Ramsay, Yeo & Jackson (1991) in their meta-analysis of dietary studies as 'too small to have any value' other groups have used it with reductions in total cholesterol of up 22% (Evans, Turner & Ghosh 1972; Jones *et al* 1982; National Cholesterol Education Program 1988).

The WHO (1990) has further advocated more recently the adoption of diets relatively rich in monounsaturated fats and with increased intakes of fruits and vegetables to help reduce the incidence of CHD. These diets approximate to those of the Mediterranean countries and the obvious attraction of such a diet is that its long term safety and indeed clinical efficacy have been tested, albeit in an uncontrolled fashion, in a natural experiment lasting more than 4000 years.

A detailed discussion of other non-pharmacological means of controlling dyslipidaemia is beyond the scope of this introduction, but they include exercise, which has been shown to increase HDL cholesterol levels (Hardman *et al* 1989), LDL apheresis, which has been used in a variety of forms to lower LDL-cholesterol levels radically (Tait *et al* 1991), and the surgical procedures of partial ileal bypass (Buchwald *et al* 1990), portacaval shunt (Starzl *et al* 1983), and, in extreme circumstances, liver transplantation (Starzl *et al* 1984). A future possibility for the management of the genetic dyslipidaemias may be manipulation of the human genome by so-called 'gene therapy'. The ethics of such procedures were resolved for British workers in a report published early in 1992 (Committee on the Ethics of Gene Therapy 1992), and after the successful transduction of hepatocytes *in vitro* from WHHL rabbits (Wilson *et al* 1988), work is now progressing to facilitate retro-virus gene transfer of the apoB/E receptor gene into the hepatocytes of individuals with FH.

## **1.6 Therapeutic Options Hypolipidaemic Drugs**

A range of lipid lowering and lipid regulating drugs is now available to the physician. Each class of drug has a unique mechanism of action although there is considerable overlap in the specific indications for their clinical use. The six main classes of drug currently used in the UK are discussed in the following sections

### **1.6.1 Bile Acid Sequestrant Resins**

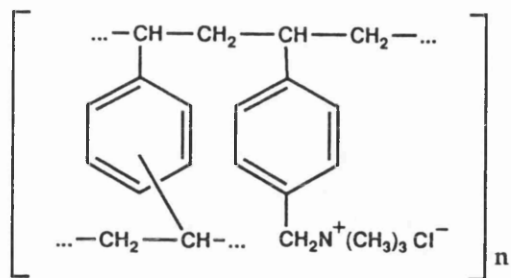
The bile acid sequestrant resin, cholestyramine, was used as a lipid lowering drug as early as 1959 (Bergen *et al* 1959). This drug, like its sister compound, colestipol can lower total cholesterol levels in the plasma by 15-30% but in practice

their efficacy is often severely limited by poor patient compliance (Lipid Research Clinics Program 1984b). The resins are marketed as insoluble powders that must be mixed with fluids such as fruit juice, or sprinkled directly on food before being swallowed. As such, many patients find them unpalatable and they are often associated with gastro-intestinal side effects such as constipation and dyspepsia.

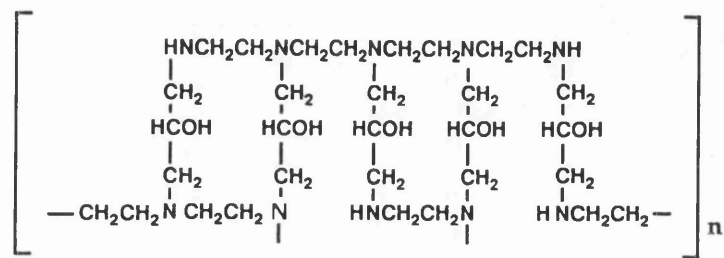
Both resins have complex structures (figure 8) and have the same mode of action, acting as non-absorbed anion-exchange resins that bind bile acids irreversibly in the intestinal lumen preventing their reabsorption in the terminal ileum. Resin-induced interruption of the enterohepatic circulation has profound effects on a number of processes regulating lipid metabolism in the liver. Normally, bile acids in the portal blood limit hepatic cholesterol oxidation by inhibiting  $7\alpha$ -hydroxylase. De-repression of this enzyme exerts a drain on regulatory liver sterol pools, which are replenished by cholesterol derived from circulating LDL particles via the apoB/E receptor. The resultant fall in LDL-cholesterol is, however, limited by an increase in hepatic cholesterol synthesis secondary to activation of the enzyme HMG CoA-reductase. Sequestrant resins also affect triglyceride metabolism in the liver, possibly by releasing bile acid induced inhibition of the pacemaker enzyme of triglyceride synthesis, phosphatidic acid phosphatase (Angelin, Bjorkhem & Einarsson 1986). As a result, secretion of triglyceride-rich VLDL increases. Most individuals can compensate by regulating VLDL metabolism accordingly, but for some this response is inadequate, and hypertriglyceridaemia persists.

The clinical efficacy of the sequestrant resins in treating hypercholesterolaemia and preventing CHD has been confirmed in the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) (Lipid Research Clinics Program 1984a, 1984b). This was a multi-centre, randomised, double-blind study involving 3806 middle-aged men with primary hypercholesterolaemia. The participants were followed for an average of 7.4 years. Those treated with cholestyramine showed an 8.5% reduction in total cholesterol, a 12.6% reduction in LDL-cholesterol, a 3% rise in HDL-cholesterol and a 4.5% rise in triglyceride, over the placebo-treated control group. These lipid and lipoprotein changes were further enhanced in those who had adhered to the prescribed 24 g. d<sup>-1</sup> dosage (Lipid Research Clinics 1984b). The drug induced lipid changes were associated with a 24% fall in definite CHD deaths and a 19% fall in non-fatal MI. Incidence rates of new positive exercise ECGs, angina and coronary artery bypass grafts were reduced by 25%, 20%, and 21% respectively in the sequestrant resin treated group. These findings provided the stimulus for other groups to investigate the clinical efficacy of lipid





CHOLESTYRAMINE



COLESTIPOL

Figure 8. Structures of the Bile Acid Sequestrant Resins.

lowering therapy, and since 1984 several major intervention trials have been published while others are currently underway.

The two sequestrant resins that are available are clinically very similar. Although some patients express a preference for one over the other, there is little for the physician to choose between them (Glueck *et al* 1972).

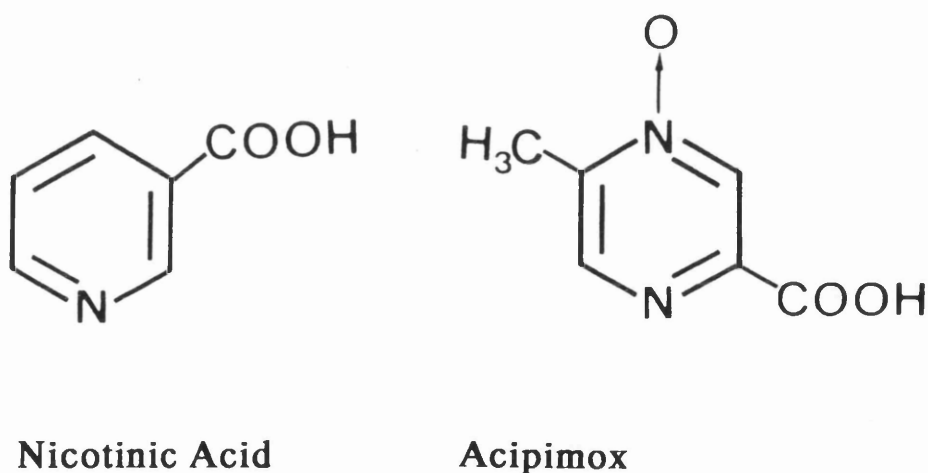
### 1.6.2 Nicotinic Acid and its Derivatives

Nicotinic acid (vitamin B3) (figure 9) is an important component of a healthy diet and is required to play a number of roles in intermediary metabolism. The recommended daily allowance of 14 mg (Committee on Medical Aspects of Food Policy 1991) provides for this but has no effect on lipoprotein metabolism. If, however, nicotinic acid is administered in pharmacological doses (i.e., grams rather than milligrams) we may observe profound changes in all lipoprotein fractions.

Nicotinic acid and its analogues have been used to treat both hypercholesterolaemia and hypertriglyceridaemia. Their clinical efficacy was first noted by Altschul,

Hoffer & Stephen (1955) and since then they have been used, with varying success in the management of all forms of hyperlipidaemia. Widespread use of nicotinic acid has been prevented, however, largely because of its disagreeable side-effects of vasodilatation and gastrointestinal irritation. These can be overcome in many patients by slowly increasing the dose over a number of weeks, and the administration of low-dose aspirin before the lipid-lowering drug is given. Nicotinic acid has had a good long term safety record and has been shown to be effective in reducing not only coronary but also total mortality in the Coronary Drug Project. In this secondary prevention trial conducted between 1966 and 1975 with five different lipid lowering drugs, nicotinic acid initially only showed a modest benefit in decreasing definite non-fatal myocardial infarctions (Coronary Drug Project Research Group 1975). However, after 15 years follow-up (almost 9 years after termination of the trial), mortality from all causes in the group given nicotinic acid during the study was 11% lower than the placebo group (Canner *et al* 1986).

Administration of nicotinic acid (2-3 g. d<sup>-1</sup>) or acipimox (750-1250 mg. d<sup>-1</sup>) (figure 9) produces sustained and significant reductions in plasma triglyceride and VLDL-cholesterol levels. Although the precise mechanism involved is still unclear, the



**Figure 9.** Structures of Nicotinic Acid and Acipimox.

effect is undoubtedly associated with a profound reduction in plasma free fatty acid levels (up to ten-fold) that is thought to reduce hepatic triglyceride synthesis by limiting substrate availability (Carlson & Oro 1962; Stirling *et al* 1985).

Adipocytes contain a hormone sensitive triglyceride lipase that in response to catecholamines increases in activity and promotes the release of free fatty acids into the bloodstream. Cyclic adenosine monophosphate (cAMP) is thought to act as a second messenger in this endocrine response. Nicotinic acid and its analogue acipimox are thought to exert their lipid lowering effect by decreasing intracellular levels of cAMP, possibly as a result of increased phosphodiesterase activity, thereby inhibiting the hormone sensitive lipase in adipose tissue (Fuccella *et al* 1980). This reduction in peripheral lipolysis leads to a decreased fatty acid flux to the liver (Stirling *et al* 1985), where it has been proposed that VLDL synthesis is consequently diminished as a result of reduced substrate supply. Acipimox has also been shown to reduce selectively hepatic triglyceride lipase activity while, in contrast to the fibrates, postheparin lipoprotein lipase activity is left largely unchanged (Stuyt *et al* 1985, Taskinen & Nikkila 1988).

The means by which nicotinic acid or its derivatives lower plasma cholesterol levels have still to be fully defined, but the most likely explanation is that it derives from the reduction in VLDL synthesis. A large proportion of the cholesterol leaving the liver does so in the form of VLDL-cholesterol. If this pathway is substantially inhibited following down-regulation of VLDL-triglyceride synthesis, then the cholesterol that would have accompanied the secreted triglyceride accumulates in the hepatocyte, perhaps leading to reduced HMG CoA-reductase activity. However, there have been several reports (Grundy *et al* 1981, Stirling *et al* 1985, Illingworth *et al* 1991) that nicotinic acid or its analogue acipimox do not affect cholesterol synthesis.

LDL is variably affected by nicotinic acid and its analogues and, as with the fibrates, its final plasma concentration is dependent on the initial hyperlipoproteinaemic phenotype (Carlson, Oro & Ostman 1968; Carlson, Olsson & Ballantyne 1977). Administration of nicotinic acid to subjects with initially high LDL levels results in a 10-15% decrease in plasma LDL. Conversely, in subjects with raised triglyceride and normal or low LDL levels, an increase in LDL concentration follows reduction in triglyceride levels by the drug. Kinetic studies (Levy & Langer 1972) suggest that nicotinic acid acts primarily to inhibit LDL

synthesis, a finding consistent with its suppressant effect on hepatic VLDL secretion.

Nicotinic acid often produces marked increases in HDL-cholesterol (Shepherd *et al* 1979c), especially in normotriglyceridaemic individuals. Concurrently there is a dramatic rise in the HDL<sub>2</sub>/HDL<sub>3</sub> ratio in the plasma. Since HDL<sub>2</sub> is considered to be cardioprotective, this is interpreted as highly desirable. The metabolic changes underlying these changes in HDL subfraction distribution have been studied in ApoAI and AII turnovers. Nicotinic acid alters the turnover rate of these apolipoproteins so that the plasma concentration of apoAI rises while that of apoAII falls (Shepherd *et al* 1979c).

More recently, nicotinic acid has been reported as having an effect on elevated Lp(a) levels. Carlson, Hamsten & Asplund (1989) using 4g. d<sup>-1</sup> showed a mean reduction of 34% in the serum levels of Lp(a) in a series of 31 patients with differing forms of dyslipidaemia.

Based on our current understanding of lipoprotein metabolism and its association with CHD risk the aggregated actions of nicotinic acid and its analogues on the lipoprotein profile should offer the recipient all-round benefit.

### 1.6.3 Probucol

Probucol (4, 4'-[(1-methylethylidene) bis (thio)] bis-[2, 6-bis (1, 1-dimethylethyl) phenol]) is structurally unique amongst lipid lowering drugs (figure 10) and the only member of its class in clinical use. It is usually given at a dose of 0.5 g b.d., which results in reductions of both LDL and HDL-cholesterol, but with little or no effect on triglyceride. The mechanism by which it achieves these effects on the lipoprotein profile are far from clear. Suggested actions include increased faecal excretion of bile acids (Beyen 1986) and inhibition of hepatic sterol synthesis (McNamara *et al* 1985). There has been renewed interest in probucol since it was noted by Parthasarathy and his colleagues (1986) that the drug's presence in LDL prevented oxidative modification of the lipoprotein. The drug has also been shown by Carew and his colleagues (1987) to reduce the progression of atherosclerosis *in vivo*. Despite these exciting observations the clinical use of probucol remains problematic. Its lipophilicity makes it difficult to study and complicates its pharmacokinetics by long term sequestration of the drug in body fat depots. Even though probucol has been shown to reduce the size of tendon xanthomata

(Yamamoto *et al* 1983) its hypoalphalipoproteinaemic effect is still regarded by many physicians as disturbing.

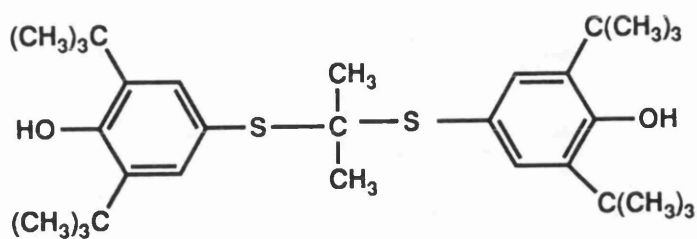
#### 1.6.4 Fibric Acid Derivatives

The fibric acid derivatives or fibrates form a family of hypolipidaemic drugs whose structure and mode of action are closely related to that of the parent compound clofibrate [Ethyl 2-(4-chlorophenoxy)-2-methylpropionate, figure 11]. Clofibrate was introduced into the UK in 1962 and found wide application as a lipid lowering agent. However, its limited efficacy and its poor benefit to risk ratio in the WHO trial (Committee of Principal Investigators 1978) have led to a steady decline in its use since 1978 (Wysowski, Kennedy & Gross 1990). Second generation fibrates, e.g. bezafibrate, gemfibrozil and more recently fenofibrate and ciprofibrate have been developed and marketed as useful agents for the treatment of hypertriglyceridaemia where they are first line therapies and, hypercholesterolemia where they are used either as first line or second line when bile acid sequestrant resin therapy fails. They also have an important place in the management of combined hyperlipidaemia (raised cholesterol and triglyceride) and are commonly used in combination regimens with the resins cholestyramine and colestipol (Goodman, Noble & Dell 1973).

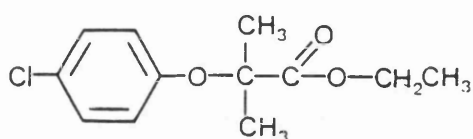
The clinical efficacy of one of the fibrates, gemfibrozil, was confirmed in the large Helsinki Heart Study (Frick *et al* 1987) where reductions in triglyceride of 35% and LDL-cholesterol by 11% and an increase in HDL-cholesterol by 11% was associated with a 34% reduction in fatal and non fatal myocardial infarctions combined.

Fibrate therapy affects all major lipoprotein fractions (chylomicrons, VLDL, LDL, and HDL) in the plasma, principally by modulating key enzymes involved in lipid metabolism. The primary action of these drugs is to reduce plasma levels of triglyceride-rich lipoproteins viz. chylomicrons and VLDL by,

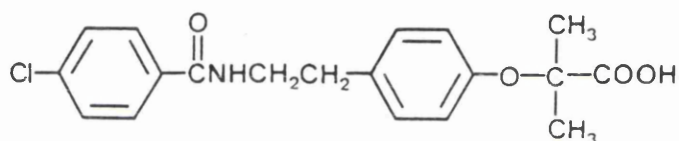
- a) stimulating the activity of lipoprotein lipase, the endothelium bound enzyme responsible for hydrolysis of triglyceride in chylomicrons and VLDL
- b) inhibiting VLDL synthesis and secretion from the liver probably as a result of the drug's effect on free fatty acid flux to the liver.



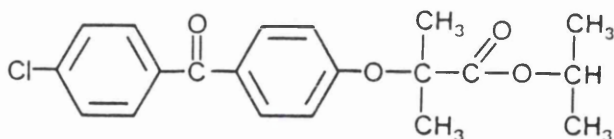
**Figure 10.** Structure of Probucol.



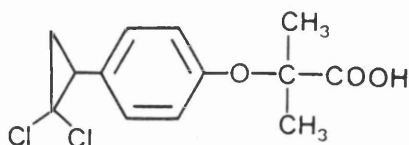
**Clofibrate**



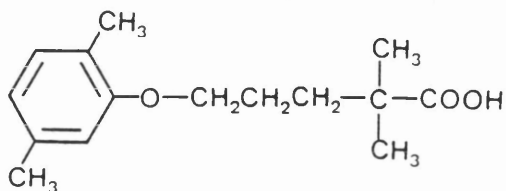
**Bezafibrate**



**Fenofibrate**



**Ciprofibrate**



**Gemfibrozil**

**Figure 11.** Structures of the Fibric Acid Derivatives.

The fibrates, therefore, reduce triglyceride levels in a consistent and readily explicable fashion. Their actions on LDL levels, however, are variable depending on the initial LDL cholesterol value. In hypercholesterolaemia where LDL levels are high, the fibrates cause a decrease in LDL, while in hypertriglyceridaemia, which is commonly associated with low plasma LDL concentrations, fibrate treatment will raise the latter (Shepherd *et al* 1984b, 1985).

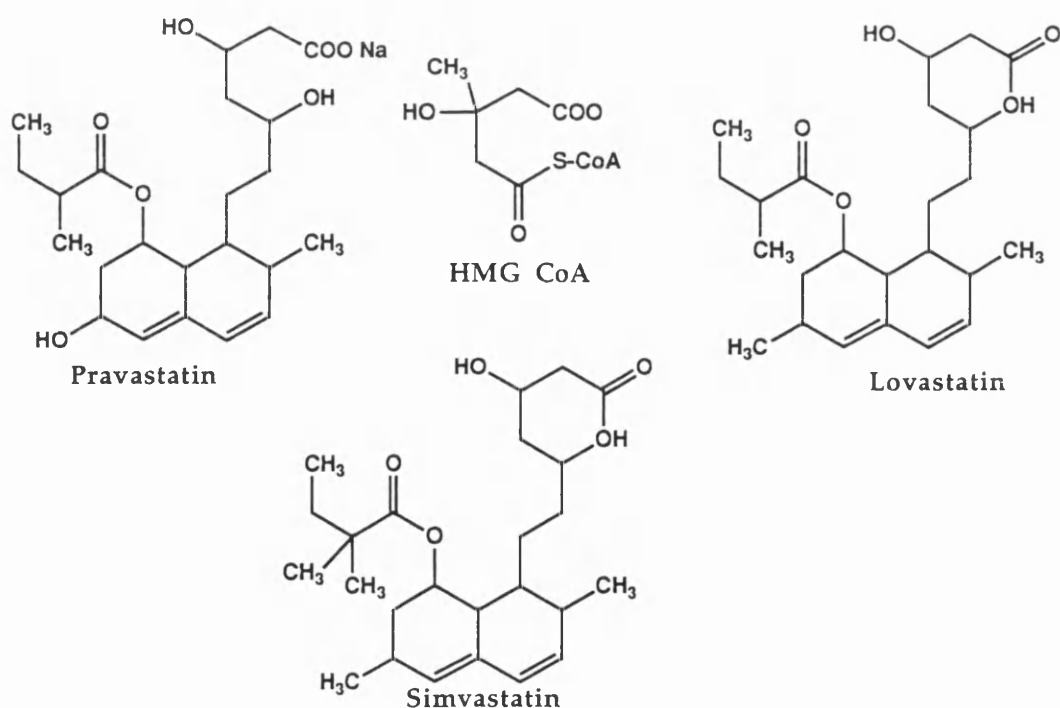
The catabolic rate of LDL has been shown to increase in hypercholesterolaemia (Stewart *et al* 1982) and decrease in hypertriglyceridaemia (Shepherd *et al* 1984b) while LDL synthetic rates in both groups remain unchanged. This apparent paradox is resolved when these changes are thought of as normalization phenomena: in hypercholesterolaemia an initially low catabolic rate is increased to normal and in hypertriglyceridaemia an initially high catabolic rate is reduced to normal.

In hypercholesterolaemia fibrate induced reductions in the atherogenic lipoprotein, LDL, are commonly accompanied by increases in the level of the anti-atherogenic lipoprotein HDL leading to important changes in the calculated atherogenic risk index. Much has been made of this overall reversal of an atherogenic profile (lowering LDL and raising HDL) and indeed the clinical use of the modern fibrates is built upon it.

#### 1.6.5 HMG CoA-reductase Inhibitors

In contrast to the fibrates, which is one of the oldest classes of lipid lowering drugs, the HMG Co A-reductase inhibitors, or 'statins' as they have come to be known, are the most recent. Interestingly, it was just over a decade from the first reports of their discovery in Japan (Endo, Kuroda & Tsujita 1976) before the Food and Drug Administration in the US approved the first compound, lovastatin, for clinical use as a lipid lowering drug, in 1987. The speed with which this transition from experimental compound to clinically approved drug has taken place is in part testament to its efficacy and, at least short-term, safety. In contrast, it has also been a cause of some alarm on the part of clinicians who see the widespread use of a group of relatively untested drugs as undesirable. The UK, however, had to wait two more years before simvastatin was launched and we have had the benefit of further clinical trials and wide US experience with the statins upon which to draw (Tobert 1988a; Illingworth, Bacon & Larsen 1988).

These drugs block endogenous cholesterol synthesis by competitively inhibiting the rate limiting enzyme HMG Co A-reductase. The structures of the three 'statins' in clinical use are shown in figure 12, alongside that of HMG CoA for comparison. All the body's cells have the capacity to synthesise cholesterol but the bulk is made in the liver. Because the liver cell is prevented from making as much cholesterol as it needs it is forced to extract an increased amount from the plasma lipoproteins. It does this by increased expression of apoB/E receptors, which bind to passing LDL particles and bring them into the cell where they are dismantled and their cholesterol load can be channelled into synthetic pathways. The corollary of this is that the plasma LDL-cholesterol level falls as the number of receptors rises and will remain suppressed as long as the drug exerts its inhibition of intracellular cholesterol synthesis. These drugs have found particular use in FH. Such patients were the first to receive the drug when the statins were launched in the US but the indications for using this class of drug have now been widened in the UK to include individuals with primary hypercholesterolaemia intolerant or not responsive to other forms of lipid lowering therapy and with total cholesterol levels in excess of 6.5 mmol. L<sup>-1</sup> (Zocor data sheet 1991). Other groups of patients such as those with



**Figure 12.** Structures of the HMG CoA-reductase Inhibitors.



nephrotic syndrome and those with diabetes mellitus have also received benefit from the powerful lipid lowering effects of the statins but their use has generally been restricted to those with primary hypercholesterolaemia (Grundy 1988).

Initial anxieties over the potential of the statins to induce lens opacities in humans have been resolved with experience. There now appears to be no increased risk of developing cataracts on these drugs (Tobert 1988a). However, the more serious possibility of life-threatening rhabdomyolysis in patients receiving statins is still regarded as real, although very rare, and seems to occur only in a small number of those individuals receiving combination therapy of statin plus fibrate or nicotinic acid or cyclosporin (Tobert 1988b). While certain combination therapies offer significant advances in the treatment of severe hyperlipidaemia the combinations described above should be avoided. Because of this potential problem, patients are instructed to report any unexplained muscle pain, tenderness or weakness and the statins should be discontinued if there is marked elevation of the muscle enzyme creatine kinase (CK). The statins are also given with caution to those individuals with impaired hepatic function and should be discontinued if the transaminases (AST and ALT) rise excessively. To facilitate this biochemical monitoring of those patients receiving statin therapy baseline measurements should be taken prior to starting therapy and rechecked in the first few months of treatment.

#### 1.6.6 Marine Oils

The fish-oil ingestion of Greenland Eskimoes has been viewed as a natural experiment testing the effects of the long chain polyunsaturates eicosapentaenoic acid (EPA, 20:5 $\omega$ 3) and docosahexaenoic acid (DHA, 22:6 $\omega$ 3) on plasma lipids and CHD. These fatty acids are found in significant quantities in cold-water fish such as salmon, tuna and mackerel, and have been refined for pharmacological use in the preparation, Maxepa®. When used in hypertriglyceridaemic patients the marine oils lower triglyceride levels by 64% in type IIb patients and an impressive 79% in type V patients (Phillipson *et al* 1985). Despite early suggestions that LDL-cholesterol levels were also lowered by marine oils, it seems that free-living patients may expect either no effect or even increases in their LDL-cholesterol levels (Harris 1989). Such an apparently adverse effect may not be a problem in the management of type V patients who have abnormally low LDL-cholesterol levels initially, but it has prevented the widespread use of marine oils in clinical practice. The apparent freedom from CHD enjoyed by fish eating populations such as the Eskimoes may

not be related entirely, or even at all, to the lipid regulating properties of the marine oils for they are reported to have other pharmacological effects (Harris 1989). Perhaps most importantly they reduce platelet aggregability and may, therefore, prevent coronary artery thrombosis (Goodnight, Harris & Connor 1981).

### 1.6.7 Combination Drug Therapy

The lipid lowering drugs are now also used increasingly in combinations for one or both of the following reasons:

- a) the therapeutic goals in hyperlipidaemic patients are continually set lower and have in some patients become unattainable on monotherapy. In the hope that two or more drugs offer an additive or even synergistic response, combination therapy has been tried.
- b) the beneficial effects of lipid lowering drug therapy are often, at least partially, offset by unwanted metabolic side-effects. These responses are sometimes simple homeostatic responses to the mechanism of drug action, e.g. bile acid sequestrant resin may de-repress cholesterologenesis or an HMG CoA-reductase inhibitor may lead to an increased translation of the enzyme protein (Fears 1983). These undesirable perturbations in lipoprotein metabolism may be limited by the dual administration of two drugs with complementary actions.

Almost every combination of hypolipidaemic therapy has been tried, often empirically, over the last twenty years. Some regimens have been highly successful in achieving their therapeutic goals, while others, such as the combination of lovastatin and gemfibrozil, are thought to be potentially detrimental. The most potent combinations are discussed below.

#### a) Bile acid sequestrant resin plus nicotinic acid

This is one of the most effective combinations for cholesterol reduction and one of the first tried (Packard *et al* 1980; Levy, Fredrickson & Schulman 1972). This regimen can reduce total and LDL-cholesterol by between 30-40%, even in patients with heterozygous FH. Many individuals are, however, unable to tolerate the side effects of nicotinic acid, which has prompted its replacement with the derivative, acipimox. This combination is reasonably well tolerated and is equally effective in reducing LDL-cholesterol levels (Series *et al* 1990).

#### b) Bile acid sequestrant resin plus fibrate

The use of fibrates in combined regimens with bile acid sequestrant resins was being examined in studies as early as 1973 (Goodman *et al* 1983), where the

original fibrate, clofibrate, was used in combination with colestipol. Although the combination produced an additional decrement in serum cholesterol the results did not achieve statistical significance. New fibric acid derivatives should, in theory, produce a better response and indeed when one of these, bezafibrate, was examined significant reductions in serum cholesterol and triglyceride were observed (Series *et al* 1989). Combination therapy with resins and fibrates is now in wide use in part because such a regimen is particularly attractive when one considers that it brings together the two therapeutic modalities shown to be independently effective in reducing coronary risk in large-scale prospective trials (Lipid Research Clinics Program 1984b; Frick *et al* 1987). Its use, however, also raises the question whether the joint administration of an anion exchange resin affects the absorption of a fibrate. To answer this question the combination of gemfibrozil and colestipol was examined by Forland, Feng & Cutler (1990) who concluded that simultaneous administration of the two drugs did indeed reduce the bioavailability of the fibrate, but added that separating the administration of the two drugs by at least two hours would obviate this interaction.

#### c) Bile acid sequestrant resin plus HMG CoA-reductase inhibitor

A combination of equal, if not greater, promise has been examined in a number of clinical studies in the US and Japan (Mabuchi *et al* 1983; Bilheimer *et al* 1983, Illingworth 1984). When bile acid sequestrant resins are administered, the hepatic bile acid pool is depleted and the hepatocytes respond by up-regulating their cell surface apoB/E receptors. This promotion of increased receptor-mediated LDL clearance is somewhat off-set by a concomitant increase in endogenous cholesterol biosynthesis. By co-administration of an HMG CoA-reductase inhibitor this effect is minimised and a greater level of apoB/E receptor activity achieved. The clinical result is a profound lowering of LDL-cholesterol by as much as 50-70%.

#### d) Fibrate plus HMG CoA-reductase inhibitor

In designing new combined regimens, a more exciting combination was thought initially to be that of fibrate plus HMG CoA reductase inhibitor. Enthusiasm for such a regimen was, however, curbed when reports of myopathy associated with lovastatin plus gemfibrozil therapy were published (Illingworth & Bacon 1989; Pierce, Wysowski & Gross 1990). Despite this, and because the combination had shown such early promise Glueck, Speirs & Tracy (1990) examined 25 patients for an average duration of 12.5 months while receiving both drugs. While mandating careful follow-up with serial creatine kinase (CK) and liver function tests, they report the combination to be safe and effective with only a 2.4% incidence of raised

CK and no symptomatic myositis. This is an important study but should not be viewed as a blanket approval of such therapy. Caution with fibrate plus HMG CoA reductase inhibitor is again urged by Illingworth & O'Malley (1990) who recently evaluated the combination of clofibrate and lovastatin in 6 type III patients and monotherapy with one or other drug in 12 patients. They showed that addition of lovastatin to clofibrate therapy or *vice versa* in patients resistant to monotherapy was of benefit in further reducing concentrations of total, VLDL and LDL cholesterol. These findings offer clinicians an alternative strategy for the management of type III hyperlipidaemia, but the authors re-emphasise the importance of close monitoring of any patients receiving such therapy.

### 1.7. General Aims of this Work

It will be clear from the above discussions that an extensive body of work has already been performed to elucidate the pathways of lipoprotein metabolism and the mechanisms of action of the various lipid lowering drugs. The present work has two aims. Firstly, to supplement this existing corpus by testing a series of mechanistic hypotheses of the pharmacological modulation of apolipoprotein B metabolism. This will be achieved by satisfying a series of related objectives. Representatives of the main lipid lowering drug classes, simvastatin, acipimox, colestipol and ciprofibrate will be examined individually and in combination. The effects of these drugs on the lipoprotein profiles of hypercholesterolaemic individuals will be reported as will their effects on the metabolism of apo B containing lipoproteins and, in the case of ciprofibrate, its effect on receptor mediated catabolism of apo-LDL. Further analysis of the effects of these drugs *in vivo* will be provided by studying their effects on the subfraction distribution of HDL and LDL, and their influence on cholesterol biosynthetic rates as evidenced by measurements of urinary mevalonate and plasma lathosterol levels: two indirect indices of this metabolic parameter. With this variety of biochemical, structural, and kinetic data it will be possible to present a general thesis on the detailed mechanism of action of the principal lipid lowering drugs.

By using a series of metabolic inhibitors, viz the lipid lowering drugs listed above, it has also been possible to assess the pathophysiology of certain aspects of lipoprotein metabolism. The large series of baseline studies presented provides a valuable source of data on the metabolic aberrations that contribute to hypercholesterolaemia in the individuals studied. Moreover, examination of the VLDL-IDL-LDL delipidation cascade using dual isotope turnover studies generates

a large volume of data on the various lipoprotein interconversions that occur in the plasma. When this is analysed in conjunction with the detailed structural data provided by lipoprotein subfraction measurements and we consider the metabolic influences of the drugs used, novel metabolic concepts are hypothesised for future testing. Thus the second aim of this work is to offer new insight into the pathophysiology of lipoprotein metabolism.

## **1.8. Nomenclature and Layout**

The results of 79 turnover experiments performed in 32 patients are presented in the following chapters. To provide a unique and simple identifier for each patient and each turnover, the following system has been adopted. All patients are coded by a number prefixed by a three letter code corresponding to their principal drug therapy, e.g. the third patient recruited in the study using simvastatin would be coded SIM 03. Each of the turnovers is identified by the appropriate patient code followed by the letter 'A' for a baseline study, 'B', for initial drug therapy and 'C' for combined therapy.

Each of the following chapters 3-9 contain details of the results of the various studies and include a discussion section appropriate to that chapter. The final chapter, 10, provides a general discussion and conclusions. For ease of reading, much of the raw data and radioactive decay curves relating to the turnover experiments have been placed in appendices 2-5.

## Chapter 2 Materials and Methods

One man is measuring the lengths of the feelers of 2,000 beetles; another the amount of cholesterol in 100 samples of human blood; each in the hope, but not in the certainty, that his series of numbers will lead him to some definite law.

John BS Haldane, 1927.

### 2.1 Study Outline

Representatives of the different hypolipidaemic drug classes were studied both as single agents and in combination. All subjects studied were defined as having primary hypercholesterolaemia but not associated with familial hypercholesterolaemia. The influence of these drugs on lipid and lipoprotein levels was examined as were the changes in lipoprotein (HDL and LDL) subfraction distribution.

Assays for the indirect assessment of *in vivo* cholesterol biosynthesis (mevalonic acid and lathosterol) were established and used to examine the effects of different lipid lowering drugs on this parameter.

Lipoprotein metabolism was followed using established protocols for the assessment of VLDL and LDL turnover at baseline and after drug therapies. The results of these studies were analysed using multi-compartmental mathematical modelling procedures.

## 2.2 Materials

All reagents used were of analytical grade and the names and addresses of all suppliers are shown in appendix 1 together with the manufacturers or suppliers of all hardware and software used in this work.

## 2.3 VLDL apoB Turnover Protocol

The metabolism of large (S<sub>f</sub> 60-400) and small (S<sub>f</sub> 20-60) VLDL was investigated following protocols previously published by Shepherd *et al* (1984b) and Packard *et al.* (1984).

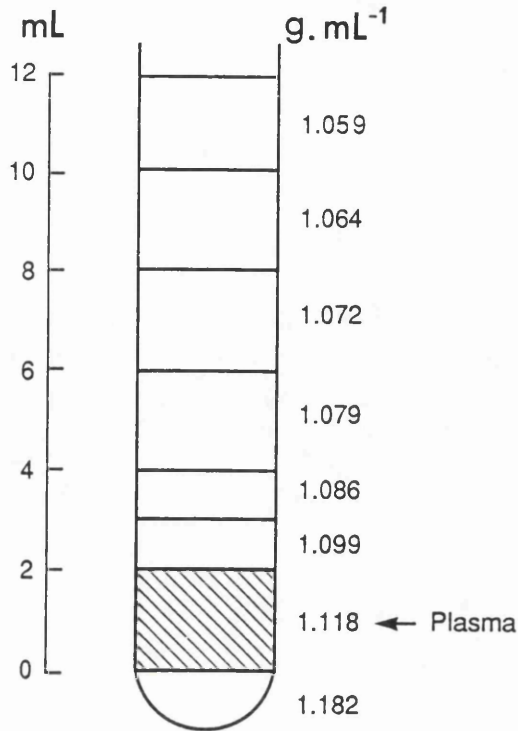
### 2.3.1 Isolation of VLDL<sub>1</sub> and VLDL<sub>2</sub>

After a 14 h fast, 250 mL of plasma were obtained from the subject by plasmapheresis. From this plasma, total VLDL ( $d < 1.006 \text{ g. mL}^{-1}$ ) was prepared by ultracentrifugation in a Beckman Ti60 rotor for 24 h at 39,000 rpm (10°C). The supernatant was collected and this VLDL solution was diluted with 0.15M NaCl to achieve a concentration corresponding to a plasma triglyceride level of approximately  $1.5 \text{ mmol. L}^{-1}$  if the subject's plasma triglyceride was initially greater than  $2.0 \text{ mmol. L}^{-1}$ . This was necessary to avoid VLDL<sub>1</sub> carryover into the VLDL<sub>2</sub> subfraction.

The density of 12mL of VLDL solution was adjusted to  $1.118 \text{ g. mL}^{-1}$  by the addition of solid NaCl ( $170 \text{ mg. mL}^{-1}$ ). A 2 mL aliquot of this preparation was layered over a 0.5 mL cushion of  $d \ 1.182 \text{ g. mL}^{-1}$  NaBr solution in six Beckman SW40 rotor tubes which had been pre-coated with polyvinylalcohol to reduce internal surface tension and permit improved layering.

A discontinuous salt gradient was constructed above each aliquot from  $d \ 1.099$ - $1.058 \text{ g. mL}^{-1}$  according to figure 13.

This set-up was centrifuged in a Beckman SW40 rotor at 39,000 rpm for 1 h 38 min (23°C) and decelerated without braking. VLDL<sub>1</sub> (S<sub>f</sub> 60-400) was removed in the top 1.0 mL of solution and the material harvested from all six tubes was pooled to yield 6.0mL of VLDL<sub>1</sub> solution. The volume removed was replaced with 1.0 mL of  $d \ 1.059 \text{ g. mL}^{-1}$  solution before continuing with the separation.



**Figure 13.** Discontinuous NaBr gradient as used for the subfractionation of apoB containing lipoproteins by ultracentrifugation

This set-up was centrifuged in a Beckman SW40 rotor at 18,500 rpm for 15 h 41 min (23°C) and decelerated without braking.

VLDL<sub>2</sub> (S<sub>f</sub> 20-60) was removed in the top 0.5 mL of solution and the material harvested from all six tubes was pooled to yield 3.0 mL of VLDL<sub>2</sub> solution.

### 2.3.2 Labelling of VLDL subfractions

Radiolabelling was performed using the iodine monochloride method (MacFarlane 1958) as modified by Bilheimer *et al* (1972). Radiolabelling of VLDL in this way will obviously introduce label into the non-B apolipoproteins and lipid components of the lipoprotein in addition to the labelling of apoB. The specificity of the procedure is only achieved by isolating apoB in the timed samples obtained after injection and thereby ensuring that only apoB radioactivity or specific activity is measured.



The iodination mixture was prepared as follows. To separate 2.0 mL aliquots of VLDL<sub>1</sub> and VLDL<sub>2</sub> solutions were added 0.5 mL of 1.0M glycine, pH 10.0 and 2.0 mCi of reductant-free Na [<sup>131</sup>I] or Na [<sup>125</sup>I].

Six µL of iodine monochloride solution (25 mM in 1.0M NaCl) were added and mixed gently. Free and bound radioiodide were separated by dialysing overnight against 3 x 2.0 L of 0.15M NaCl, pH 7.4 (4°C).

These ratios of iodine/protein result in the incorporation of no more than one atom of iodine per molecule of apoB in VLDL.

### **2.3.3 Sterilisation and Measurement of Specific Activity**

The labelled tracers were sterilised by membrane filtration through 0.45 µm filters (Acrodisc, Gelman Sciences) which had been primed with the subject's native plasma. This was done immediately prior to re-injection into the subject.

The radioactivity concentration (µCi. mL<sup>-1</sup>) was calculated after sterilisation by counting a 10 µL aliquot of labelled VLDL and comparing with [<sup>131</sup>I] and [<sup>125</sup>I] simulated standards.

### **2.3.4 Subject Preparation**

In all studies it was essential to ensure that thyroidal uptake of radioiodide had been blocked by the oral administration of potassium iodate (170mg twice daily). This regimen was commenced three days prior to injection of radiolabelled lipoproteins and was continued for the next 28 days. The turnover studies were conducted on an out-patient basis and all subjects were instructed to adhere strictly to their regular diet and lifestyle. This was done to ensure steady state conditions for the investigation of lipoprotein metabolism. Informed consent was obtained from all subjects prior to the study.

### **2.3.5 Injection and Sampling Protocol**

On the third day after plasmapheresis the subject was admitted at 08.00 h following an overnight fast and the following procedure was observed.

An indwelling cannula was placed in a peripheral vein to facilitate repeated venous blood sampling. This was flushed with sterile 0.15M NaCl to maintain patency.

Autologous [ $^{131}\text{I}$ ]-VLDL<sub>1</sub> and [ $^{125}\text{I}$ ]-VLDL<sub>2</sub> were injected in rapid sequence into a peripheral vein in the opposite arm. These injectates were separated and chased with boluses of sterile 0.15M NaCl.

10 mL venous blood samples were collected via cannula at the following time points post-injection: 10 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 14 h and thereafter 10 mL fasting venous samples were obtained each morning for the next 12 days. All samples were collected into tubes containing K<sub>2</sub>EDTA as anti-coagulant to give a final concentration of 1 mg. mL<sup>-1</sup>.

To minimise chylomicron production the subject remained fasting for the first 10 h of the study but was allowed unlimited non-caloric fluids.

### 2.3.6 Reisolation of Tracer

Plasma was obtained from each of the blood samples by low speed centrifugation, 1000g (4°C).

From 2.0 mL aliquots of plasma VLDL<sub>1</sub> and VLDL<sub>2</sub> were isolated as above (2.3.1)

In addition IDL (S<sub>f</sub> 12-20) and LDL (S<sub>f</sub> 0-12) were isolated by subjecting the loaded SW40 rotor to two further centrifugation steps of 39,000 rpm for 2 h 35 min (23°C) and 30,000 rpm for 21 h 10 min (23°C). IDL was removed in the top 0.5 mL of the gradient and LDL in the top 1.0 mL.

ApoB was precipitated by adding an equal volume of freshly redistilled 1,1,3,3-tetramethylurea (TMU) at 37°C to each lipoprotein fraction (Kane *et al* 1975). This mixture was vortexed immediately and allowed to stand at 37°C for 1 h.

The resulting insoluble pellicle of lipid and apoB was separated by centrifugation at 1000g for 30 min. The TMU-soluble phase was removed carefully with a thinly drawn out glass Pasteur pipette and the tube inverted to draw off any remaining liquid.

The apoB pellet was delipidated by extracting with organic solvents (ethanol:diethyl ether 3:1 v/v) overnight at -20°C. The protein pellet was reformed by further low speed centrifugation and the solvent removed.

The pellet was dried with a diethyl ether wash for 2 h at -20°C and after removal of this ether the samples were placed uncapped in an incubator at 40°C for 10 min or until all solvent had evaporated.

The isolated apoB was redissolved by adding 1.0 mL of 0.5M NaOH and the samples were left at 37°C overnight or until dissolution was complete.

### 2.3.7 Calculation of Pool Sizes

VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL and LDL were prepared by cumulative flotation ultracentrifugation of 6 x 2 mL aliquots of pooled plasma obtained on four occasions during the course of the 13 day VLDL-turnover study.

Total protein and TMU-soluble protein were measured in each lipoprotein fraction by the methods of Lowry *et al* (1951) and Kane *et al* (1975) respectively.

ApoB concentrations were calculated as the difference between total protein and TMU-soluble protein and expressed as mg. 100 mL<sup>-1</sup>

The apoB concentration in each lipoprotein fraction was additionally determined by averaging the total protein measurements in each TMU precipitate from every time point throughout the study. This latter procedure was initially used as a check of the TMU-soluble method but because of its greater reliability it was adopted as the principal method in the majority of the VLDL apoB turnovers.

Pool sizes for apoB in the four lipoprotein fractions were derived from the product of plasma volume and the plasma concentration of apoB in each fraction. The subject's plasma volume was estimated as 4% of the body weight. This was considered appropriate as none of the subjects studied were obese or oedematous.

Correction of the apoB pool sizes for centrifugal losses was made by comparing the  $\text{VLDL}_1 + \text{VLDL}_2 + \text{IDL} + \text{LDL}$  cholesterol recovered from the cumulative flotation ultracentrifugation, to the 'non-HDL' cholesterol measured in the  $\beta$ -quantification of plasma as described in section 2.5.1. In most subjects the cholesterol recovered using cumulative flotation was 80-90% of the mean 'non-HDL' cholesterol throughout the turnover period. The apoB pool sizes were therefore adjusted by an appropriate multiplication factor to take into account these losses.

### 2.3.8 Data Collection and Handling

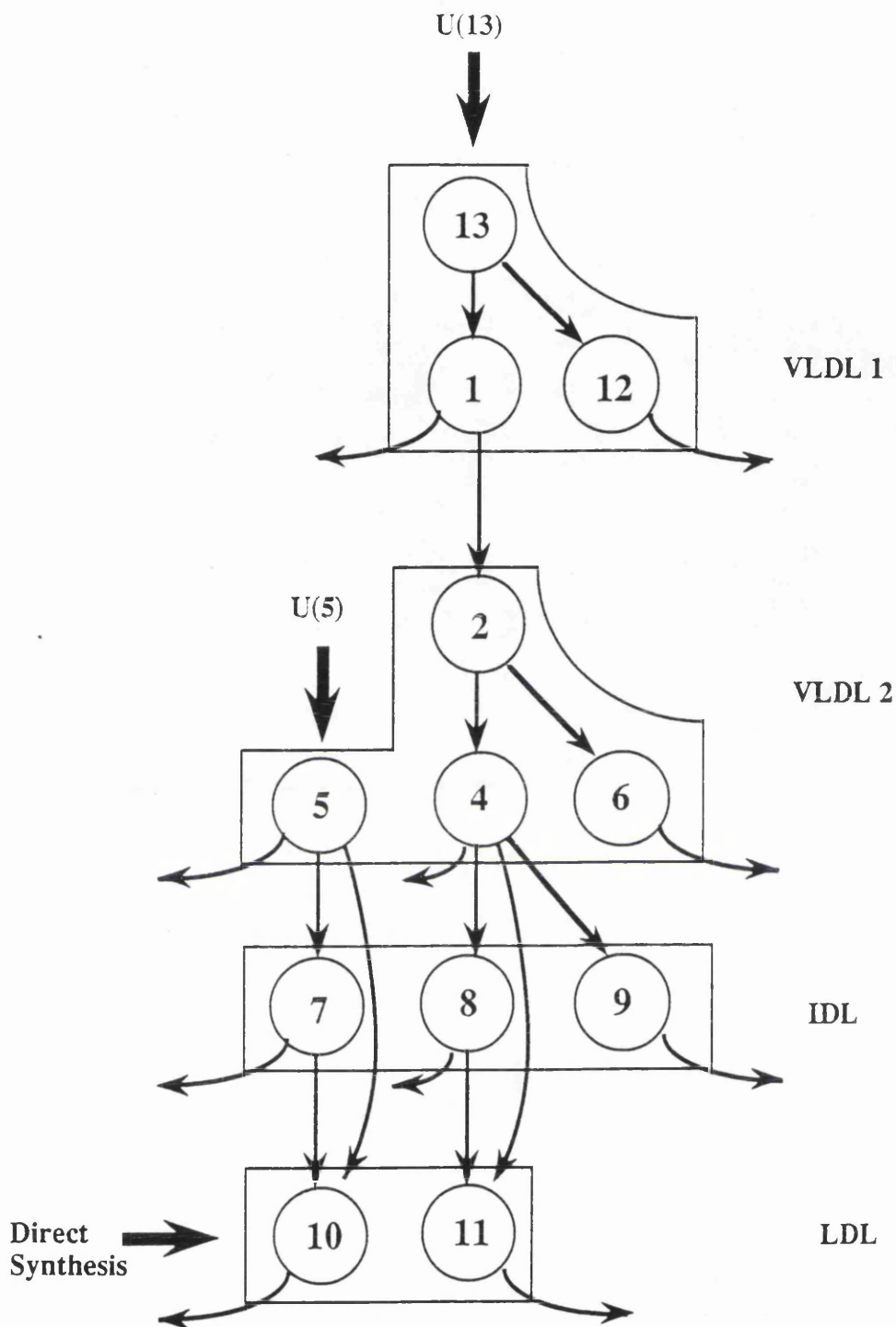
$[^{131}\text{I}]$  and  $[^{125}\text{I}]$  were counted in each of the apoB samples prepared from the lipoprotein fractions in an automated gamma counter (Packard) for 1.0 min.

To calculate specific activities, the protein content of each sample was measured using a modification of the method described by Lowry *et al* (1951) in which the NaOH is omitted from the Cu/alkali reagent. The NaOH added to the apoB protein to facilitate its dissolution gave the appropriate pH for the final Lowry reaction mixture. The procedure was further modified to allow for large throughput of samples and duplicate analysis. This is further described in section 2.5.3.

### 2.3.9 Data Analysis

The apoB specific activities were multiplied by the average pool size to generate total radioactivities. These were then expressed as a fraction of the total apoB radioactivity present 10 min after injection. These data were used to construct apoB decay curves for each lipoprotein fraction.

These curves and the apoB masses associated with each lipoprotein fraction were used to simulate apoB metabolism using a multi-compartmental model computed by the SAAM 30 modelling program (Berman & Weiss 1974) on the Glasgow University VAX system. The metabolic model used is shown in figure 14 and is essentially that described elsewhere (Packard *et al* 1984, Demant *et al* 1991a) with the exception that two further sub-compartments have been added in  $\text{VLDL}_1$  to take account of the second, slower exponential which is seen in some individuals.



**Figure 14.** Multicompartmental model for apolipoprotein B metabolism in VLDL<sub>1</sub> (S<sub>f</sub> 60-400), VLDL<sub>2</sub> (S<sub>f</sub> 20-60), IDL (S<sub>f</sub> 12-20) and LDL (S<sub>f</sub> 0-12).  $U(13)$  and  $U(5)$  represent *de novo* input of apoB into VLDL<sub>1</sub> and VLDL<sub>2</sub> respectively. Direct synthesis into the LDL density interval was calculated as the difference between the absolute catabolic rate of apoB in this fraction (observed mass x overall FCR) and the input from VLDL<sub>2</sub> and IDL.

The main features of the model were as follows:

- a) apoB direct input may occur at the level of VLDL<sub>1</sub>, VLDL<sub>2</sub>, and LDL. Input of newly synthesised apoB into the Sf 20-60 density range (compartment 5) was required because firstly not all the Sf 20-60 apoB mass can be accounted for by transport from large VLDL and secondly when large and small VLDL are labelled separately the kinetics of appearance of these tracers in IDL and LDL apoB are different. Usually the radioactivity derived from labelled small VLDL appeared more quickly in these denser fractions and accounted for a higher proportion of their mass. Provision for this phenomenon was made by incorporating in the model parallel pathways for VLDL<sub>1</sub> and VLDL<sub>2</sub> and their metabolic products appearing in IDL and LDL. *De novo* synthesis of apoB into the LDL density range was permitted to explain the discrepancies between observed and calculated LDL apoB metabolism. Some consequences of this allowance are discussed in chapters 3 and 10.
- b) VLDL is delipidated in a stepwise manner following the concept of Berman *et al* (1978).
- c) slowly catabolized, remnant pools are postulated in VLDL<sub>2</sub> and IDL density ranges ( compartments 6 and 9 respectively)
- d) LDL was distributed between two plasma pools, to take account of the different metabolic properties of LDL derived from VLDL<sub>1</sub> (compartment 11) or VLDL<sub>2</sub> (compartment 10).

This model provided an acceptable fit for the observed data. The calculated rate constants were defined with a fractional standard deviation (FSD) of less than 0.06. For VLDL<sub>1</sub>, VLDL<sub>2</sub> and IDL, the calculated masses derived from the kinetic analyses were within 10% of the observed values in almost every case. With LDL, however, the observed apoB pool was consistently greater than the calculated mass. Reasons for this discrepancy are discussed with the results below.

## 2.4 LDL Turnover Protocol

The technique used to assess LDL kinetic parameters was basically the same as that originally developed by Langer *et al* (1972) with the modification described by Shepherd *et al* (1979a), which allows the measurement of receptor-mediated and receptor-independent LDL catabolism.

### 2.4.1 Isolation of LDL

After a 14 h fast, 50 mL of plasma were obtained from the subject.

LDL (d 1.030-1.050 g. mL<sup>-1</sup>) was prepared by rate zonal ultracentrifugation according to the method of Patsch *et al* (1974).

The collected lipoprotein fraction was de-salted by dialysing against 0.15M NaCl/0.01% Na<sub>2</sub>EDTA (pH 7.0) and concentrated by pressure filtration through an XM 100 A cellulose membrane (Amicon) to a protein concentration of 3-5 mg. mL<sup>-1</sup>, as determined by the method of Lowry *et al* (1951).

### 2.4.2 Labelling of LDL

Radiolabelling was carried out by the iodine monochloride method of MacFarlane (1958) as modified by Shepherd, Bedford & Morgan (1976).

The following iodination mixtures were prepared:

Two 1.0 mL aliquots of LDL solution were mixed separately with 0.5 mL of 1.0M glycine, pH 10.00 and 2.0 mCi of either reductant-free Na [<sup>131</sup>I] or Na [<sup>125</sup>I].

An appropriate volume of iodine monochloride solution (25 mM in 1.0M NaCl) was added to yield an ICl:protein ratio of approximately 20 mol:500,000 Da of protein, and mixed gently. This procedure introduces an iodine atom into tyrosine residues at a level of less than 10 mol iodine/molecule of LDL protein.

Bound and free radio-iodide were separated by passing the the two iodination mixtures over separate 1.0 x 10 cm columns of Sephadex G-25 (PD10 column, Pharmacia). The eluting buffer used was 0.15M NaCl containing 0.01% Na<sub>2</sub>EDTA (pH 8.1).

### 2.4.3 CHD Modification

1.0 mL [<sup>131</sup>I]-LDL at a protein concentration of 3-5 mg. mL<sup>-1</sup> in a solution of 0.15M NaCl containing 0.01% Na<sub>2</sub>EDTA was added to 2.0 mL of freshly prepared 0.15M 1,2-cyclohexanedione (CHD) in 0.2M sodium borate buffer, pH 8.1.

This mixture was incubated in a water bath at 35°C for 2 h.

Modified LDL was separated from unbound reagents by passing the mixture over a 1.0 x 10 cm column of Sephadex G-25 (PD10 column, Pharmacia) and eluting with 0.15M NaCl containing 0.01% Na<sub>2</sub>EDTA (pH 8.1).

#### **2.4.4 Sterilisation and Measurement of Radioactivity.**

The labelled tracers were sterilised immediately prior to re-injection by membrane filtration through separate 0.22 µm filters (Acrodisc, Gelman Sciences) which had been primed with the subject's unlabelled LDL.

The radioactivity concentration (µCi. mL<sup>-1</sup>) was determined after sterilisation by counting 10 µL aliquots of CHD-modified [<sup>131</sup>I]-LDL and native [<sup>125</sup>I]-LDL and comparing with <sup>131</sup>I and <sup>125</sup>I simulated standards.

#### **2.4.5 Subject Preparation**

Thyroidal uptake of radioiodide was blocked as described in 2.3.4. As with the VLDL turnovers, the LDL turnover studies were conducted on an out-patient basis and all subjects were instructed to adhere strictly to their regular diet and lifestyle. This was done to ensure steady state conditions for the investigation of lipoprotein metabolism. Subjects received detailed instruction on the collection of 24 h urine specimens. Informed consent was obtained from all subjects prior to the study.

#### **2.4.6 Injection and Sampling Procedures**

Because the initial collection of fasting blood was done at 08.00 h it was possible to have the labelled modified and native LDL aliquots ready for re-injection within the same working day.

Autologous CHD modified [<sup>131</sup>I]-LDL and Native [<sup>125</sup>I]-LDL were injected in rapid sequence into a peripheral vein. These injectates were separated and chased with boluses of sterile 0.15M NaCl.

After a time lapse of 10 min the first venous blood sample was collected from a peripheral vein in the opposite arm of the subject. Thereafter, a 10 mL fasting



venous blood specimen was collected each morning for the next 14 days. All blood specimens were collected into tubes containing K<sub>2</sub>EDTA as anti-coagulant to give a final concentration of 1 mg. mL<sup>-1</sup>.

Continuous 24 h urine collections were collected timed to 08.00 h over the 14 day turnover period to estimate urinary excretion rates and urinary:plasma radioactivity ratios.

#### 2.4.7 Calculation of Pool Sizes

LDL was prepared from serial 4.0 mL fasting plasma samples obtained on days 3, 7, 10 and 13 of the turnover study by ultracentrifugation at limit densities d 1.010-1.063 g. mL<sup>-1</sup>.

Each LDL sample was dialysed against 0.15M NaCl and total protein and TMU soluble protein were measured by the methods of Lowry *et al* (1951) and Kane *et al* (1975) respectively

The apoB concentration in each sample was calculated as the difference between total and TMU-soluble protein and expressed as mg. 100mL<sup>-1</sup>. In order to correct for centrifugal losses, recovered radioactivity was related to that seen in plasma.

A pool size for apo-LDL was derived from the product of plasma volume (obtained by isotope dilution) and the mean apo-LDL plasma concentration.

#### 2.4.8 Data Collection and Handling

Plasma was obtained from each fasting venous blood sample by low speed centrifugation (4°C).

<sup>131</sup>I and <sup>125</sup>I were counted in 2.0 mL aliquots of each sample in an automated gamma counter (Packard) for 10 min.

<sup>131</sup>I and <sup>125</sup>I were counted in 4 mL aliquots of each, well-mixed 24 h urine collection. Urine volumes were determined and thus total daily urine radioactivities. The completeness of these 24 h urine collections was assessed by measurement of 24 h urinary creatinine excretion.

Plasma radioactivity decay curves were constructed and urine:plasma ratios were calculated for each time point.

### 2.4.9 Data Analysis

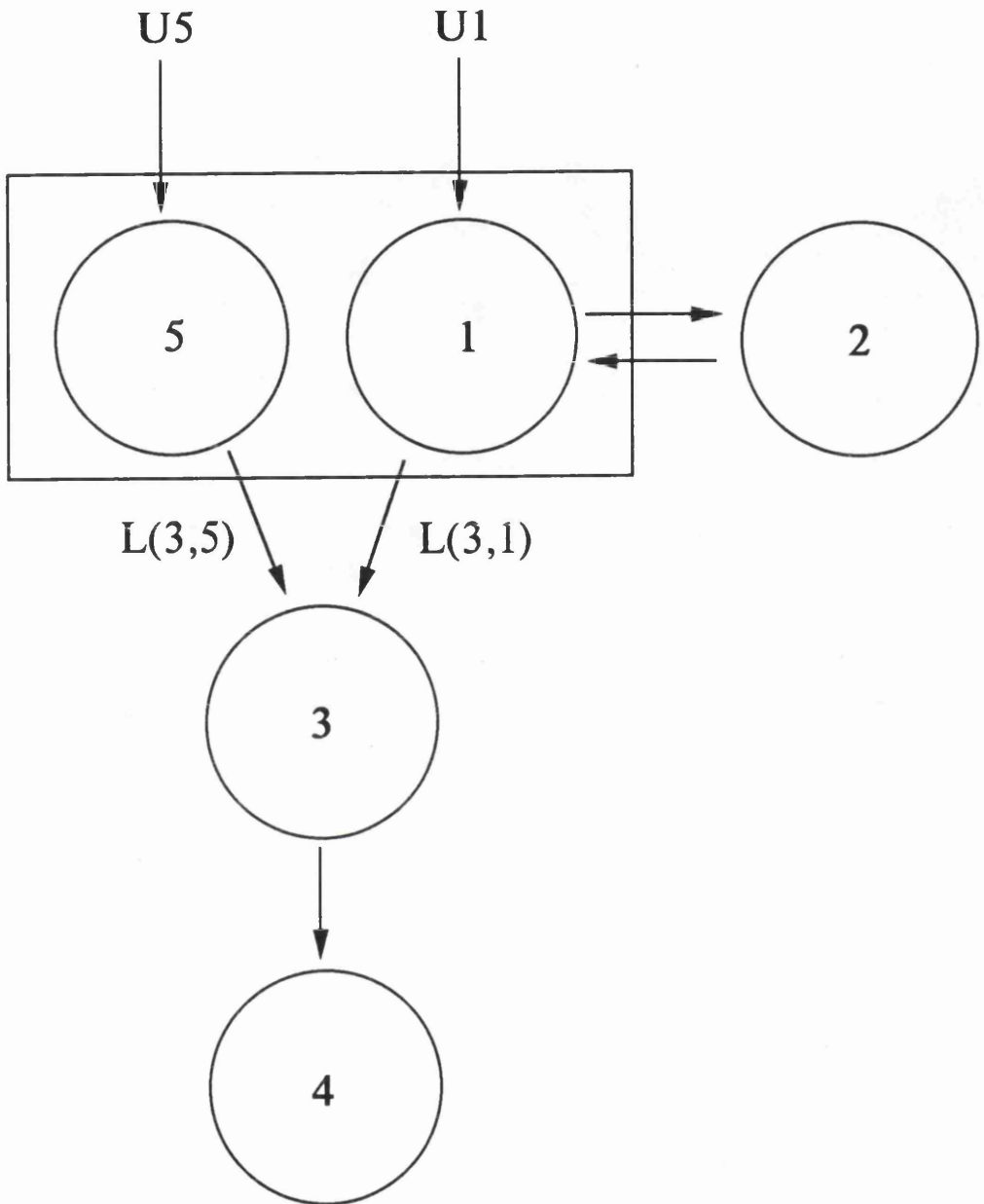
Plasma  $^{131}\text{I}$  and  $^{125}\text{I}$  radioactivity data were analysed by the procedure of Matthews (1957) as adopted by Shepherd *et al* (1979a) to determine FCRs for total, receptor-mediated and receptor-independent apo-LDL catabolism. The rate of elimination of  $^{125}\text{I}$ -labelled native apo-LDL represented total catabolism of this fraction while that of  $^{131}\text{I}$ -labelled CHD modified tracer was used as a measure of receptor-independent removal. The difference between these two FCRs (native and CHD) therefore provided an index of the activity of the receptor pathway. The urine/plasma radioactivity ratios for both native and CHD-modified apo-LDL were calculated from the urinary output of  $^{131}\text{I}$  or  $^{125}\text{I}$  in a 24 h period and the plasma radioactivity at the beginning of that period. Since there was approximately a half-day delay between the clearance of radioactivity from the plasma and its appearance in urine (Boston, Greif & Berman 1982; Foster *et al* 1986), the latter value represents the relevant mean plasma radioactivity for the 24 h urine collection. The synthetic rate for apo-LDL was calculated as the product of the apo-LDL circulating mass and the fractional catabolic rate. This was expressed per kilogram body weight for each subject. Additionally, plasma and urine radioactivities were analysed together by multi-compartmental modelling using the SAAM 29 (Berman & Weiss 1974). With this approach, previously described by Boston *et al* (1982) and Foster *et al* (1986), LDL was not treated as a single homogeneous entity, but as shown in figure 15, two plasma pools, one of which was in equilibrium with an extravascular pool, were required for the simultaneous fitting of plasma and urine radioactivity data (Caslake *et al* 1992).

## 2.5 Lipids, Proteins and Lipoproteins.

These were assayed using a variety of chemical, enzymatic, centrifugal and immunoassay techniques as described in detail below.

### 2.5.1 B Quantification

Lipoprotein classes were prepared by a combination of ultracentrifugation and selective precipitation, using standard methodologies (Lipid Research Clinics Program 1974). VLDL were separated as a floating fraction. The infranatant was then treated with heparin/ $\text{Mn}^{2+}$  (at a final concentration of  $1.3 \text{ g. mL}^{-1}$  heparin and  $0.092\text{M Mn}^{2+}$ ) to precipitate LDL and leave HDL in solution



**Figure 15.** Model of Apo-LDL Metabolism. Apo-LDL in the plasma is divided into two compartments, A and B. Pool A (compartment 5) is more readily cleared from the plasma. Pool B (compartment 1) exchanges with the extravascular space (compartment 2). The model is similar to that previously used by Boston *et al* (1982) and Foster *et al* (1986).  $U(5)$  and  $U(1)$  represent input rates for pools A and B respectively.  $L(3,5)$  and  $L(3,1)$  are elimination rate constants for the 'fast' and 'slow' pools. Compartment 3 is the body iodide pool, which is cleared into the urine (compartment 4) at a fixed rate of 2.5 pools.  $d^{-1}$ .

(Warnick & Albers 1978). The cholesterol content of whole plasma, of the top (VLDL), and bottom (LDL and HDL) fractions, and of the heparin/ $\text{Mn}^{2+}$  supernatant (HDL) were measured as described below.

#### *Ultracentrifugation.*

Five mL of plasma was placed in a Beckman Ultra-clear tube (13x64 mm) and overlaid with 2 mL of  $d\ 1.006\ \text{g. mL}^{-1}$  solution. Tubes were capped and centrifuged overnight at 35,000 rpm ( $4^{\circ}\text{C}$ ) in a Beckman 50.4 rotor, then sliced 25 mm from the top and the supernatant collected into a 3 mL flask. The contents of the bottom fraction were transferred to a 5.0 mL volumetric flask, the tube washed with saline, the wash added to the flask, and the volume adjusted to 5.0 mL with 0.15M NaCl.

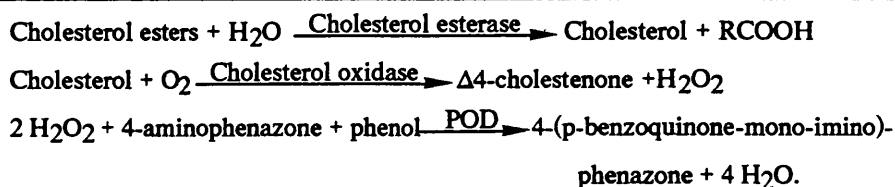
#### *Precipitation of LDL.*

An aliquot (1.0 mL) of the bottom fraction was placed in a Beckman centrifuge tube and 50  $\mu\text{L}$  of precipitating reagent [9.56 g  $\text{Mn Cl}_2 \cdot 4\ \text{H}_2\text{O}$  + 1.05 g (approximately  $5 \times 10^5$  units) heparin sodium salt (Sigma London Chemical Co.) in 25 mL 0.15M NaCl] were added and mixed. The mixture was kept at  $4^{\circ}\text{C}$  for 15 min then centrifuged at 10,000 rpm for 30 min, and the supernatant separated immediately for cholesterol analysis.

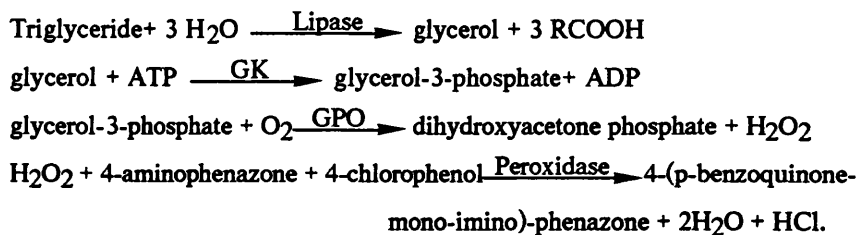
### 2.5.2 Compositional Analyses

Total cholesterol and triglyceride were determined in whole plasma and in lipoprotein preparations by enzymatic colorimetric assays on a Hitachi 704 auto-analyser.

Total cholesterol was assayed using Boehringer Kit No. 704121 the principal of which is shown below:



Triglyceride was assayed using Boehringer Kit No. 704113 the principal of which is shown below:



The resultant colour changes were measured at 505 nm.

Free (unesterified) cholesterol and phospholipid were determined by enzymatic colorimetric assays on a Centrifichem Encore centrifugal analyser (Baker Instruments).

Free cholesterol was estimated with Boehringer Kit No. 310328 in which the cholesterol esterase above is omitted from the enzyme reagents. Esterified cholesterol was calculated by difference.

Phospholipid was estimated with Boehringer Kit No. 691844, which employs an enzymatic, colorimetric assay dependent on the liberation of choline by phospholipase D.

### 2.5.3 Modification of Lowry Protein Assay

Protein measurements were performed according to the method of Lowry *et al* (1951). Because of the number of samples to be processed in this study the Lowry protein assay as originally described was tedious, expensive in terms of time, reagents and consumables and often, because of the required sample size, did not permit duplicate analyses. The latter point was particularly of concern in view of the importance of obtaining accurate results to calculate specific activity data for the turnover timepoints and apoB pools sizes. For these reasons it was decided to evaluate the adaptation of the current method to microtitre plate technology. Exactly the same reagents were used but the volumes of reagents and samples were scaled down accordingly and the resultant colour changes were read using a microtitre plate scanner, which in addition, generated a standard curve and computed the final protein concentrations.

*Reagents*

## i) Stock Reagents:

Soln A 2%  $\text{Na}_2\text{CO}_3$  in 0.1N NaOHSoln B 2% Na K Tartrate in  $\text{H}_2\text{O}$  (deionised)Soln C 1%  $\text{Cu SO}_4$  in  $\text{H}_2\text{O}$  (deionised)Soln D 2%  $\text{Na}_2\text{CO}_3$  in  $\text{H}_2\text{O}$  (deionised)

Folin-Ciocalteu Reagent (BDH)

## ii) Working Reagent:

Biuret. To 10 mL Soln A (or Soln D, if VLDL turnover samples) 0.1 mL of Soln B and 0.1 mL of Soln C were added. To clarify turbid samples, e.g. VLDL, Sodium Dodecyl Sulphate ( $1\text{mg. mL}^{-1}$ ) was added to the working Biuret reagent (Curry *et al* 1978). Failure to add SDS yielded erroneously high results in these samples.

*Standards*

i) Stock standard. Human Serum Albumin  $1\text{mg. mL}^{-1}$ . Stored in aliquots at  $-20^\circ\text{C}$

ii) Working standards. A working standard curve in the range 0-300  $\mu\text{g. mL}^{-1}$  was prepared in duplicate by taking 0, 2, 4, 6, 8 and 10  $\mu\text{L}$  of stock standard and adjusting the final volume to 40  $\mu\text{L}$  with deionised water as shown below:

Vol of Stock Std ( $\mu\text{L}$ )	Vol of Deionised $\text{H}_2\text{O}$ ( $\mu\text{L}$ )
0	40
2	38
4	36
6	34
8	32
10	30

When analysing VLDL turnover samples, 10  $\mu\text{L}$  of 2N NaOH were used in place of 10  $\mu\text{L}$  of  $\text{H}_2\text{O}$ . Appropriate aliquots of sample (VLDL<sub>1</sub> 40  $\mu\text{L}$ , VLDL<sub>2</sub> 30  $\mu\text{L}$ , IDL 15  $\mu\text{L}$ , LDL 5  $\mu\text{L}$ ) were taken and the final volume adjusted to 40  $\mu\text{L}$  with deionised water (or 0.5N NaOH if VLDL turnover samples).

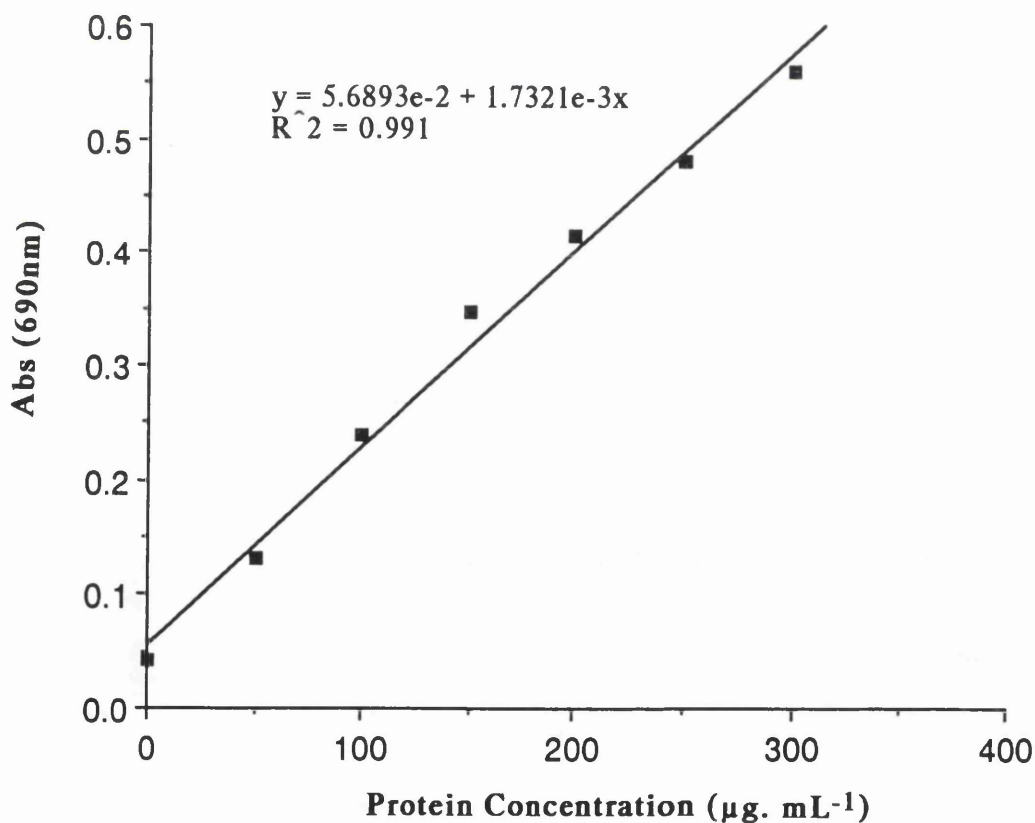
*Quality Control*

Bovine Serum Albumin  $0.15\text{mg. mL}^{-1}$  stored at  $-15^{\circ}\text{C}$ .  $10\mu\text{L}$  of the control material was taken and the final volume adjusted to  $40\mu\text{L}$  with deionised water.

*Procedure*

- 1) To  $40\mu\text{L}$  of standard, control or sample dilution in a 96 well microtitre plate (Sterilin, UK) were added  $200\mu\text{L}$  working Biuret and mixed.
- 2)  $20\mu\text{L}$  of working Folin-Ciocalteu reagent (diluted 1:1 v/v with deionised water) were added and mixed immediately.
- 5) Absorbances read on Dynatech MR5000 Microtitre Plate Reader (Dynatech Labs Ltd, West Sussex, UK) at  $690\text{nm}$ .
- 6) Equation of regression line of absorbance vs standard concentrations computed and unknowns calculated. An example of the standard curve obtained is shown in figure 16.

Std Curve Micro-Lowry APX 06C



**Figure 16.** Example of Standard Curve used in Microtitre Lowry Assay.

**Characterisation of Micro Lowry Protein Assay**

Accuracy:	Comparative determinations with existing Lowry Protein Assay (n=24), r=0.98	
Precision:	a) Within batch (n=25)	CV=7%
	b) Between batch (n=12)	CV=9%
Sensitivity:	0.06 mg. mL <sup>-1</sup>	
Costing:	The micro assay represented an 80% saving in reagents, a 70% saving in consumables, and depending on the operator, a saving in technical time of 50-75 %.	

The micro-Lowry protein assay was acceptable in terms of accuracy and precision and because it afforded the possibility of performing duplicate analyses it was used for all protein estimations in this study with the exception of TMU soluble proteins which because of their organic solvent content had to be performed in glassware.

**2.5.4 Analytical Ultracentrifugation**

HDL subfraction masses were estimated by Mrs Dorothy Bedford (Institute of Biochemistry, Glasgow Royal Infirmary). Plasma concentrations of HDL<sub>2</sub> and HDL<sub>3</sub> were estimated by analytical ultracentrifugation in a Beckman Model L8-70 ultracentrifuge equipped with an ultraviolet scanning attachment, (Beckman Instruments) using an AnF rotor with double sector centrepiece (Shepherd *et al* 1984a).

**2.5.5 LDL Subfraction Analysis**

The LDL subfraction distribution in the patients presented here were estimated by Dr Bruce Griffin and Mrs Muriel Caslake (Institute of Biochemistry, Glasgow Royal Infirmary) using non-equilibrium density gradient centrifugation as described by Griffin *et al* (1990). Briefly, fresh plasma was fractionated into three distinct LDL subfractions on a six-step, curvilinear salt gradient that was subjected to 24 h centrifugation in a Beckman SW40 rotor (40,000 rpm, 23°C). After centrifugation, the LDL subfractions were eluted by upward displacement using a heavy density solution and detected by continuous monitoring of absorbance at 280nm. This procedure generated LDL subfraction profiles from which it was possible to resolve three subfractions that corresponded in size and density to those described by Krauss (1987a), namely



LDL-I, LDL-II and LDL-III. The individual subfraction areas beneath the LDL profile were quantified using Beckman 'Data Graphics' software (Beckman, U.K.). The integrated areas, after being adjusted by specific extinction coefficients calculated previously for LDL-I, II and III, were expressed as fractions of total LDL mass by proportionating the lipid and protein mass of total LDL (d 1.019-1.063 g. mL<sup>-1</sup>). This resulted in concentration values for each LDL subfraction in milligrams of lipoprotein per 100 mL of plasma (Griffin *et al* 1990).

### 2.5.6 Apolipoprotein E Phenotyping

Apolipoprotein E phenotyping was performed on all patients by Mrs Dorothy Bedford and Mrs Linda McCusker (Institute of Biochemistry, Glasgow Royal Infirmary) using methods as described by Menzel & Utermann (1986) and Havekes *et al* (1987). To detect apoE isoforms, 10 µL of plasma were delipidated, redissolved in 6M urea containing 0.1M TRIS pH 10.0, 5% 2-mercaptoethanol and 1% sodium decyl sulphate and applied to a vertical polyacrylamide slab gel of 5% (w/v) acrylamide in 8M urea containing 1% ampholyte (pH range 4-6). The cathode buffer was 0.2M NaOH and the anode buffer 0.1M H<sub>3</sub>PO<sub>4</sub>. Isoelectric focussing was carried out at 3W per gel for 16h. Transfer by electrophoresis from the acrylamide slab gels to nitrocellulose membranes was performed as described by Towbin, Staehelin & Gordon (1979). The transfer buffer contained 0.2M glycine, 0.025M TRIS and 20% methanol and the current applied was 0.4A for 3 h. ApoE isoforms bound to the membrane were visualised by immunostaining using a monoclonal or polyclonal apoE-specific antibody and an appropriate IgG-binding second antibody linked to horseradish peroxidase.

### 2.5.7. Lipoprotein (a)

Plasma lipoprotein (a) [Lp(a)] was determined using the commercial kit, Innatest Lp(a) (Innogenetics SA, Belgium), by Mrs Linda McCusker or Mrs Elizabeth Murray (Institute of Biochemistry, Glasgow Royal Infirmary). This assay consists of a solid phase mouse monoclonal anti-Lp(a), and a sheep anti-apoB polyclonal second antibody, which is labelled with the enzyme horseradish peroxidase. This labelled antibody binds to any solid phase antibody/Lp(a) complex because it can bind to the apoB moiety of the Lp(a) complex. Further incubation with enzyme substrate produces a blue colour,

which turns to yellow when the reaction is stopped with sulphuric acid. The intensity of the colour formed is proportional to the amount of Lp(a) in the original sample.

## **2.6. Mevalonic Acid**

In order to investigate the effects of lipid lowering drug therapy on the cholesterol biosynthetic rate an assay for urinary mevalonic acid was established based on that in the laboratory of Dr D. Roger Illingworth in Portland, Oregon, USA.

### **2.6.1 Background**

Mevalonic acid is present in both human urine and plasma (Popjak *et al* 1979) and previous work has already established that both plasma and urinary concentrations of mevalonate parallel changes in hepatic and whole body cholesterol biosynthesis and therefore may serve as an index of hepatic HMG CoA reductase activity (Kopito & Brunengraber 1980; Kopito *et al* 1982; Parker *et al* 1982; Parker *et al* 1984).

### **2.6.2 Purification of Mevalonate Kinase from Porcine Liver**

The purification of this enzyme was performed by the author in the laboratory of Dr D. Roger Illingworth under the supervision of Dr Anu Pappu and with the help of Ms Shannon Magennis. The method used was that of Levy & Popjak (1960) as modified by Popjak *et al* (1979) and Pappu (unpublished). It involved the preparation of a crude extract of porcine liver by protamine and ammonium sulphate fractionation which was further purified by affinity and hydroxylapatite chromatography.

#### ***Initial Extract***

Approximately 5kg of parasite free pig liver were obtained from the abattoir immediately after slaughter of the animal and were placed on ice. Working at 4°C the liver was trimmed of fat and finely minced in a rust-free meat grinder. Each 600g batch of liver mince was extracted with 1L of sucrose solution (0.35M sucrose containing 35 mM KHCO<sub>3</sub> and 1mM disodium EDTA, pH 7.5). This mixture was stirred gently for 90 seconds and centrifuged at 2000g

for 30 minutes (4°C) yielding approximately 1L of initial extract in the supernatant. This gentle and brief extraction procedure minimises the amount of protein and therefore ATPase extracted.

#### *Protamine Precipitation*

To each litre of initial extract, 75mL protamine solution (protamine sulphate from salmon sperm, 10mg. mL<sup>-1</sup>, Sigma) was added to achieve a final concentration of 0.7mg. mL<sup>-1</sup>. After mixing for 2 minutes at 4°C the mixture was centrifuged at 2000g for 30 minutes, 4°C. This step increased the specific activity of mevalonate kinase in the preparation 3-4 fold.

#### *Ammonium Sulphate Precipitation*

Finely powdered ammonium sulphate was slowly added to the protamine supernatant to achieve 30% saturation at 4°C. The mixture was stirred gently until the salt dissolved while pH was monitored and maintained at approximately 7.5, by the addition of NH<sub>4</sub>OH if necessary. After 30 minutes the small precipitate formed was removed by centrifugation at 12,000 g for 30 minutes (4°C). More ammonium sulphate was added to the supernatant to achieve a 45% saturation and mixed for a further 30 minutes. The precipitate was collected by centrifuging at 12,000g for 30 minutes (4°C). After removing the supernatant, the precipitate was resuspended in an equal volume of an 80% saturated ammonium sulphate solution containing 20mM potassium phosphate buffer, 1mM disodium EDTA and 5mM 2-mercaptoethanol, pH 7.5.

#### *Red Sepharose Affinity Chromatography*

Affinity adsorption and elution of mevalonate kinase was carried out at room temperature using a glass column 25 x 4.4 cm i.d., with a polypropylene support net (Amicon) packed with 200mL of Red Sepharose (Amicon Matrix Gel Red A). Those sites on the Red Sepharose capable of irreversibly binding mevalonate kinase were first inactivated by circulating the column with 2L of 0.1% bovine serum albumin (BSA) overnight. Excess BSA was washed out with 2L of 1M ammonium sulphate in 10mM potassium phosphate buffer and 1mM disodium EDTA, pH 7.5. Two litres of wash buffer (10mM potassium phosphate buffer with 1mM disodium EDTA, and 5mM 2-mercaptoethanol pH 7.5) were passed through the column by gravity. The ammonium sulphate preparation above was centrifuged at 12,000 g for 30 minutes (4°C) and the resulting pellet resuspended up to 1L in wash buffer. This sample preparation was loaded onto the column by gravity in 5 cycles. Unbound protein was

eluted with 2L of wash buffer and mevalonate kinase was eluted with 1L of 0.5M ammonium sulphate solution in 10mM potassium phosphate buffer with 2mM ATP, 1mM Mg Cl<sub>2</sub>, and 5mM 2-mercaptoethanol, pH 7.5. This eluate was concentrated down overnight to approximately 25 mL under nitrogen at 172kPa, using a Diaflo® ultrafiltration membrane, PM10 (Amicon). The concentrate was centrifuged at 12,000g for 30 minutes, 4°C to remove solids and the supernatant was dialysed overnight at 4°C with two changes against 1mM potassium phosphate buffer with 1mM disodium EDTA, and 5mM 2-mercaptoethanol pH 7.5, to remove ammonium sulphate and to dilute the potassium phosphate buffer present.

### *Hydroxylapatite Purification*

This procedure was carried out at 4°C and all buffers used were prepared in CO<sub>2</sub> free deionised water and adjusted to pH 7.5 at 4°C. The column was set up by packing 2.5 x 60cm glass column with Bio-gel HT hydroxylapatite (Bio-rad) in 0.5 mM potassium phosphate buffer. Before purification the column was equilibrated with 1L 1mM potassium phosphate buffer with 1mM disodium EDTA and 5mM 2-mercaptoethanol. The dialysed sample was loaded onto the column by gravity and the column was washed with a further 100mL of equilibration buffer above. Mevalonate kinase was eluted by a linear gradient generated from 1L each of 1mM and 200mM potassium phosphate buffers containing 1mM disodium EDTA, and 5mM 2-mercaptoethanol pH 7.5. The gradient flowed at 0.5 mL/minute and 12 mL fractions were collected overnight. The protein content of each tube was determined by the protein assay of Lowry *et al* (1951) and the enzyme activity present was estimated using the assay described below in 2.6.4. The 3-4 fractions of highest specific activity were pooled as were those on either side of this peak which also contained significant amounts of mevalonate kinase activity. Each pool was adjusted by adding 10mg BSA and made up to 2mM disodium EDTA, 2mM dithiothreitol and ammonium sulphate, 80% saturation. The desired final specific activity of the stored enzyme was approximately 5  $\mu\text{mol. mg}^{-1}. \text{min}^{-1}$ .

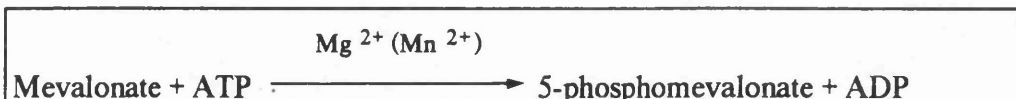
At each stage in the purification procedure the specific activity and % yield were checked using the mevalonate kinase assay described below (2.6.3).

### 2.6.3 Spectrophotometric Assay of Mevalonate Kinase

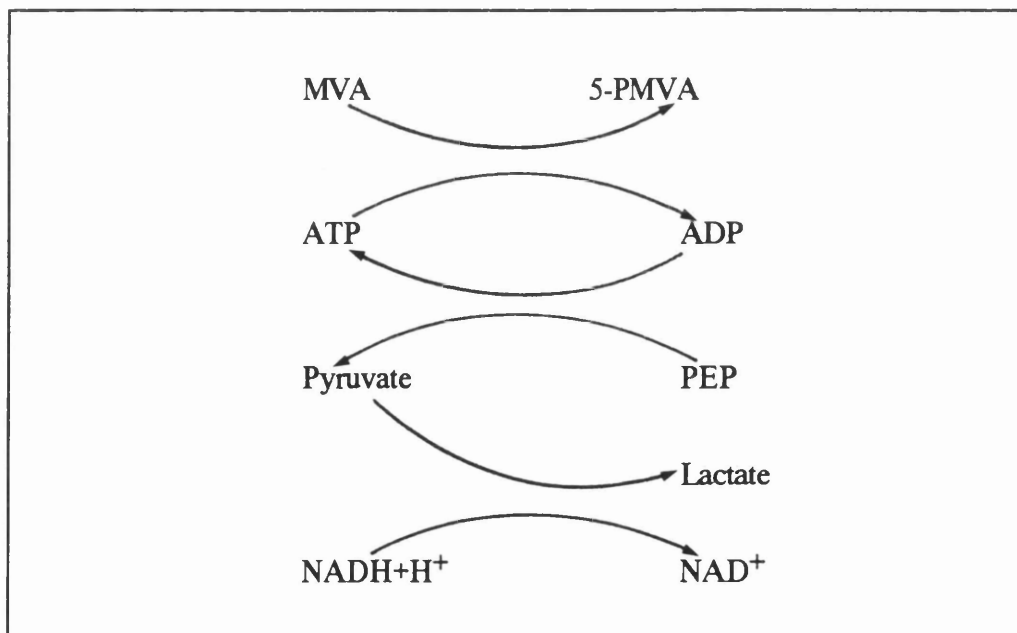
#### Mevalonate Kinase

(ATP: Mevalonate 5-phosphomevalonate EC 2.7.1.36)

This enzyme was first isolated from yeast extracts (Tchen 1958) and was subsequently discovered in animal tissues (Markley & Smallman 1961). It is known to be abundant in mammalian liver (Popjak 1969). The enzyme catalyses the following reaction:



and has been found to be stereospecific for the R-enantiomer of mevalonic acid (Cornforth, Cornforth & Popjak 1962). Hepatic mevalonate kinase may be assayed by the spectrophotometric method of Levy and Popjak (1960). This involved coupling the kinase reaction with a pyruvate/LDH system as shown in figure 17 and observing the associated change in absorbance at 340nm.



**Figure 17.** Coupled Reactions used in the Assay of Mevalonic Acid

*Assay Procedure*

Working close to the spectrophotometer the following reagents and water were mixed in a 10mL glass test tube:-

Reagent	Volume (mL)
Distilled water	2.24
TRIS- HCl Buffer 0.3M, pH 7.4.	2.00
Mg Cl <sub>2</sub> 0.3M	0.10
KF 1M *	0.06
ATP 0.20M, pH 7.0	0.20
PEP 50mM, pH 7.0	0.10
BNADH (disodium salt, grade III ) (reconstituted with 2.13mL of 0.01 M KHCO <sub>3</sub> .)	0.40

\*KF is only required for the assay of the 'initial extract' and the 'protamine supernatant' in order to inhibit ATPase.

The tube was then stored in the ice bucket until required. A batch of approximately 5 tubes was prepared in this fashion in advance.

Next the following were added:

Reagent	Volume (mL)
Cysteine hydrochloride 0.6M	0.10
KOH 1M	0.10
LDH/PK solution (5000 units of each dissolved together in 4mL of 1% albumin solution.)	0.10

0.60 mL of sample was added and mixed.

The reaction mixture was split into 2 x 2.9mL aliquots in 2 quartz spectrophotometer cuvettes with path length 1cm. To the reference cuvette 0.1mL distilled water was added and placed in the spectrophotometer. To the other cuvette 0.1mL 0.1M Potassium RS-mevalonate was added and the cuvette inverted immediately to mix. Because the reaction may proceed to completion very rapidly the cuvette was placed in the spectrophotometer and reading commenced as quickly as possible. To avoid obtaining a negative curve the

normal reference/sample arrangement in the spectrophotometer was reversed. The absorbance was read at 340nm for 3 minutes and recorded at 10 second intervals. As a reagent check ADP solution (2.5mM, pH 7.0) was added in place of the mevalonate.

To obtain Potassium RS-mevalonate for use in this assay, 130mg mevalonolactone was dissolved in 2mL of 1M K OH and 4mL of distilled water, and the solution heated to 50°C for 30 mins. The pH of the cooled solution was carefully adjusted to 7.3 with 0.1M HCl and the volume made up to 10mL with distilled water.

Definitions used in the calculation of mevalonate kinase activity were as follows:

One unit of enzyme activity equals the amount of enzyme which when assayed spectrophotometrically, phosphorylates mevalonate at an initial rate of 1  $\mu$ mol per minute at 25°C

Specific activity equals units of enzyme activity/mg protein.

#### **2.6.4 Preparation of Samples for Mevalonic Acid Assay**

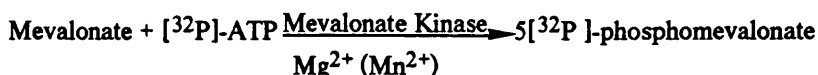
Twenty-four hour urine collections were obtained. The completeness of the collection was assessed by patient questioning and measurement of 24 hour urinary creatinine excretion. Aliquots (3 x 5 mL) were taken from the collection, centrifuged at 1000g (4°C) for 30 mins to remove any solids. Aliquots of the supernatant (1mL) were frozen at -20°C. These aliquots are known to be stable for at least 3 years (DR Illingworth, personal communication).

#### **2.6.5 Radioenzymatic Assay of Mevalonic Acid**

The assay of mevalonic acid was performed according to the radioenzymatic method of Popjak *et al* (1979) as modified by Illingworth *et al* (1989).

The assay involves phosphorylation of mevalonate with radiolabelled  $^{32}\text{P}$ -ATP using the enzyme mevalonate kinase to yield radiolabelled 5-phosphomevalonate as shown below. This product is isolated by ion exchange chromatography along with [ $^{14}\text{C}$ ] labelled 5-phosphomevalonate which has been added in known amount to the reaction mixture. The ratio of  $^{32}\text{P}/^{14}\text{C}$  in

the 5-phosphomevalonate collected is a linear function of the initial mevalonic acid content of the samples.



### *Preparation of Triethylammonium Carbonate Buffer 2M*

In a fume cupboard, 275mL triethylamine (BDH) was added to approximately 600mL of distilled water in a 1L bottle. The bottle was placed in an ice bucket and crushed ice packed around it. CO<sub>2</sub> was bubbled through the solution at a pressure of 3.45kPa for approximately 2.5h until the pH comes down to 9.7. The stock 2M TEAC buffer was then made up to 1L with distilled water and stored at 4°C for no more than 7 days.

### *Dialysis of Stock Enzyme (Mevalonate Kinase)*

The stock enzyme was stored at 4°C. Approximately 0.03 units of enzyme was required per sample. Therefore, the required volume for each run was calculated, depending on the specific activity of the enzyme batch.

Stock enzyme solution (0.3-0.5mL) was taken as appropriate and placed in a small plastic eppendorf tube and centrifuged at 12,000g (4°C) for 30 minutes. The supernatant was carefully removed leaving behind a yellow/green pellet. This pellet was redissolved in 300μL of Potassium Phosphate Buffer 20mM + 1mM DTT (Sigma) (dialysing buffer) and the resulting enzyme solution dialysed against 500 mL of the above buffer overnight with two changes, using pre-soaked Spectrapor tubing, 12-14,000 MWCO, 25mm wide.

### *Preparation of Standards*

Stock Potassium RS-mevalonate was prepared from mevalonolactone as described above in 2.6.2. From stock frozen 0.1M solution a 1μM solution was prepared by dilution and stored in 1 mL aliquots at -20°C. It should be noted that 1μL of standard contains 1pmol of a racemic mixture of potassium mevalonate and therefore only contains 0.5pmol of R-enantiomer. Since mevalonate kinase as noted above in 2.6.4 is stereospecific this is an important point for the final calculation of results.



*Preparation of Samples*

Prepared samples and standards were thawed in water bath at 37°C. and the following volumes added to labelled plastic centrifuge tubes:

Urine	20 $\mu$ L
Standards	0, 20, 50, 100 $\mu$ L

and made up to 100 $\mu$ L with freshly prepared potassium phosphate buffer 20mM, pH 7.4.

*Preparation of Incubation Mixture*

The following reaction mixture was prepared:

Potassium phosphate buffer 1M (pH 7.4) Stock.	750 $\mu$ L
Mg Cl <sub>2</sub> 0.3M	250 $\mu$ L
ATP solution *	8 $\mu$ L
Mevalonate kinase + buffer †	1385 $\mu$ L

\* ATP (Boehringer Mannheim MV602), 4.9mg in 315 $\mu$ L Potassium phosphate buffer 20 mM.

† Contents of dialysis tubing were taken and the tubing rinsed with the unused dialysis buffer to obtain appropriate volume.

Behind plexiglas and lead shielding <sup>32</sup>P-ATP (NEN 2.0mCi. mL<sup>-1</sup>) 100 $\mu$ L was added to this mixture and vortexed immediately. Four microcuries were required for each sample, therefore the exact volume of labelled ATP added depended on its specific activity.

*Incubation*

Fifty microlitres of the above reaction mixture were added to each tube and allowed to stand at room temp. for at least 2.5 h behind shielding. The reaction was stopped by adding 25 $\mu$ L concentrated. HCl (10M) to each tube in rapid succession. The following were then added in the correct sequence to each tube:

- i) 30 $\mu$ L (approx. 0.06 $\mu$ Ci) [<sup>14</sup>C]-5-phosphomevalonic acid.(Amersham, 25 $\mu$ Ci in 12.5 mL H<sub>2</sub>O).
- ii) 25  $\mu$ L ATP/ADP (0.1152g ATP+0.0160g ADP in 750 $\mu$ L of 0.5M K H<sub>2</sub>PO<sub>4</sub>
- iii) Ethanol 0.9mL to precipitate ATP/ADP (labelled and unlabelled).

The tubes were vortexed and left on ice behind plexiglas shielding for 30 minutes. Samples were transported to the centrifuge in a plexiglas box and centrifuged at 1000g (4°C) for 10 minutes.

After centrifugation, behind plexiglas and lead shielding, the supernatant was carefully removed from each tube and added to pre-labelled tubes containing 5mL of 0.1M TEAC buffer. This transfer was carried out using a new glass pasteur pipette for each sample and standard and great care was taken not to take up any of the precipitate from the pellet or from the walls of the centrifuge tube.

### *Ion-Exchange Chromatography*

Ten samples were run in each assay using the custom built fraction collector pictured in figures 18 & 19 The specifications of this device are listed below:

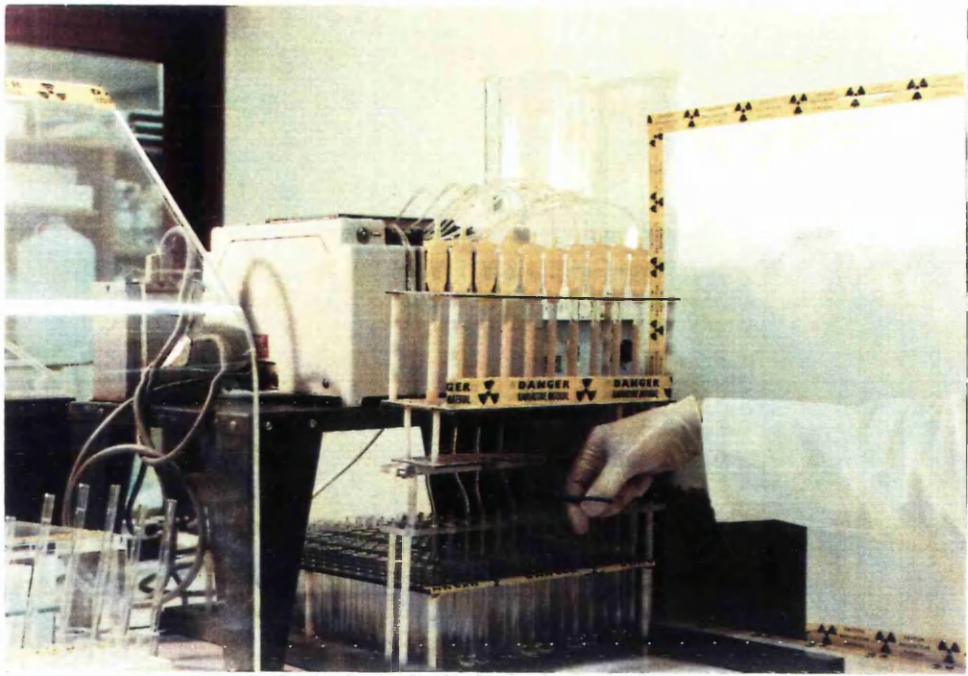
- i) 10 x Econo-columns (0.7 x 10cm) and caps (Bio Rad).
- ii) 12 channel peristaltic pump. (Technicon) Flow rate 0.6 mL/ minute.
- iii) Gradient maker, capacity 2L. (Isolab)

The anion exchange resin used was AG® 1-X8 200-400 mesh chloride form (Bio Rad) This was converted to the carbonate form according to the procedure described by Popjak *et al* (1979) Briefly, this involved washing newly purchased resin repeatedly with methanol and water and cycling with 1M H Cl and 1M Na OH. It was then treated with 2M Na<sub>2</sub> CO<sub>3</sub> over several days and then washed with water. It was then equilibrated in a large column for several days with 20mM triethylammonium carbonate buffer, pH 9.7. After equilibration the resin was removed from the column and kept at 4°C as an approximately 50% (w/v) slurry in 20mM TEAC buffer. The resin had to be regenerated in a similar fashion after each assay.

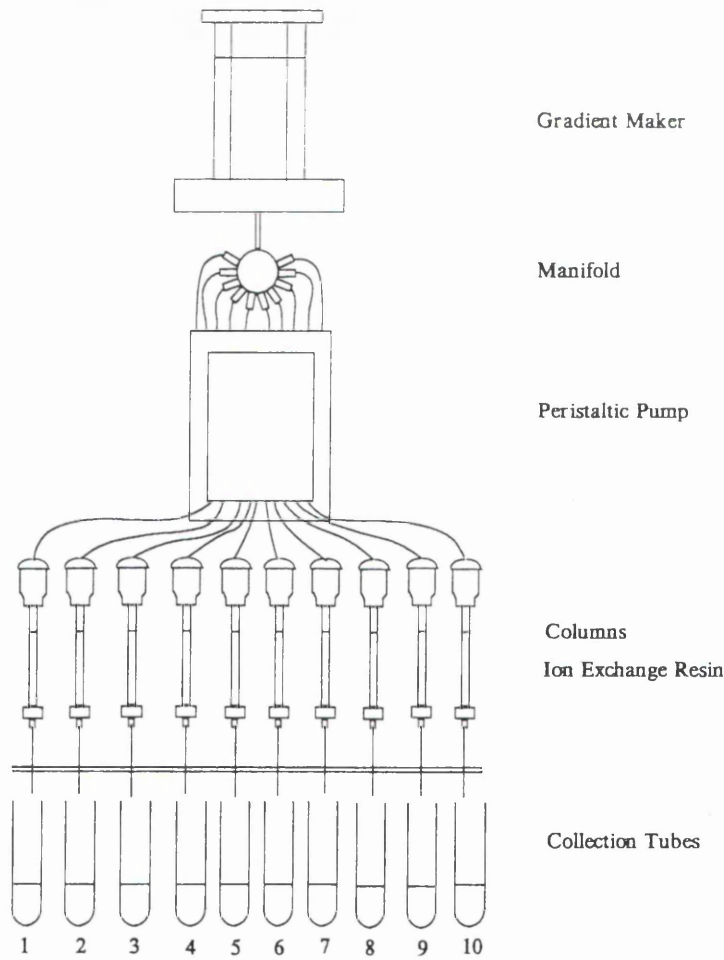
### *Loading Column*

The columns were filled with approximately 5mL of regenerated resin and allowed to settle making sure that all columns had the same height of resin Once equilibrated the columns were loaded with samples and a linear TEAC gradient was pumped through.

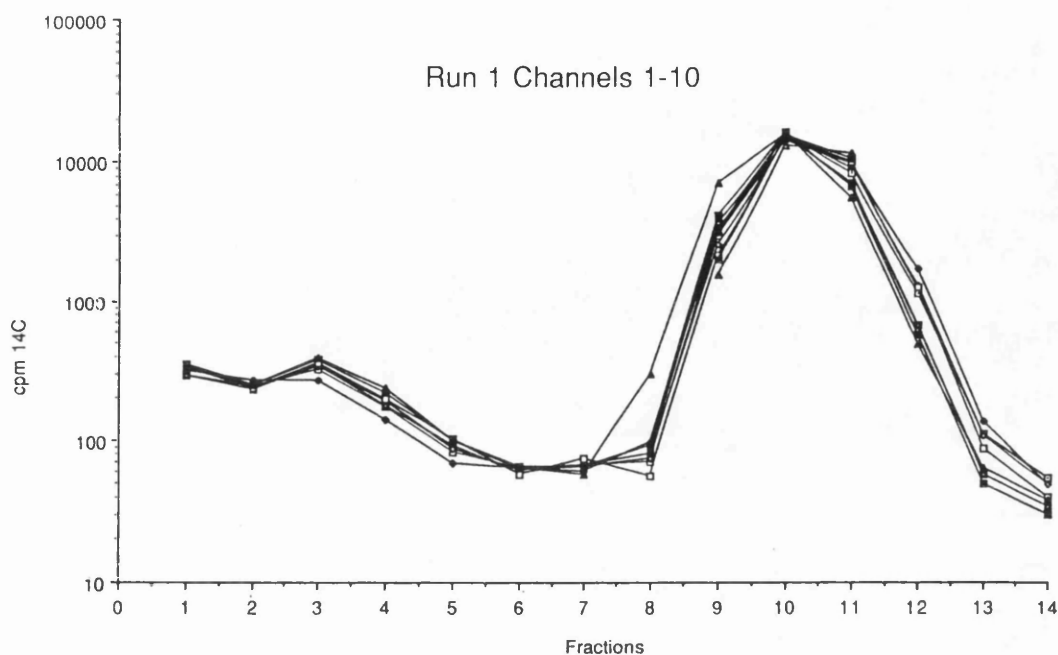
To check the uniformity of the columns in this custom built fraction collector sample blanks containing [<sup>14</sup>C]-5-phosphomevalonic acid were run. A composite graph showing the simultaneous elution of [<sup>14</sup>C]-5-phosphomevalonic acid in all 10 channels is illustrated in figure 20.



**Figure 18.** Photograph showing the custom built fraction collector used for the assay of mevalonic acid.



**Figure 19.** Schematic diagram of the custom built fraction collector used for the assay of mevalonic acid.



**Figure 20.** Uniformity Check of The 10 Channel Fraction Collector. Sample blanks spiked with  $[^{14}\text{C}]$ -5-phosphomevalonic acid were run and the  $^{14}\text{C}$  counted in each 3 min fraction from each column. This composite graph shows that the peak fractions for all ten columns are eluted simultaneously.

### *Collecting Fractions*

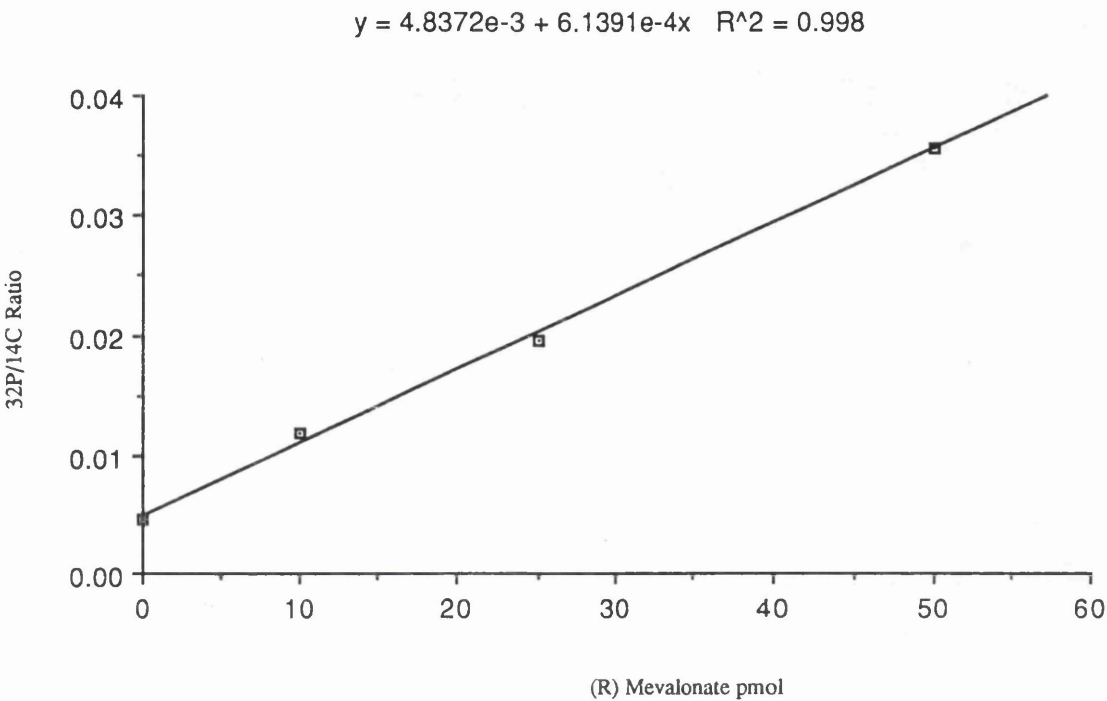
As the gradient was pumped through the columns the fractions collected in the first 20 minutes were discarded and fractions were then collected at 3 minute intervals. The appropriate two peak fractions for each column were then transferred to counting vials (Super polyethylene scintillation vials 20 mL, Canberra Packard). 2.5 mL of each fraction was added to each vial containing 10 mL of scintillation fluid (Ultima-Gold, Canberra Packard).  $^{32}\text{P}$  and  $^{14}\text{C}$  were counted in the fractions for 10 minutes each on an automated  $\beta$ -counter (Packard).

The procedure was found to have an inter-assay coefficient of variation of  $<5\%$ .

### *Calculation*

The mean  $^{32}\text{P}/^{14}\text{C}$  ratio of the two peak fractions was taken and the blank subtracted. The mean ratio was read from a standard curve (example shown in figure 21) to obtain answers in  $\text{pmol} \cdot 20\mu\text{L}^{-1}$ . These were adjusted to take account of the 24h urine volume and expressed as  $\mu\text{mol} \cdot 24\text{h}^{-1}$ .

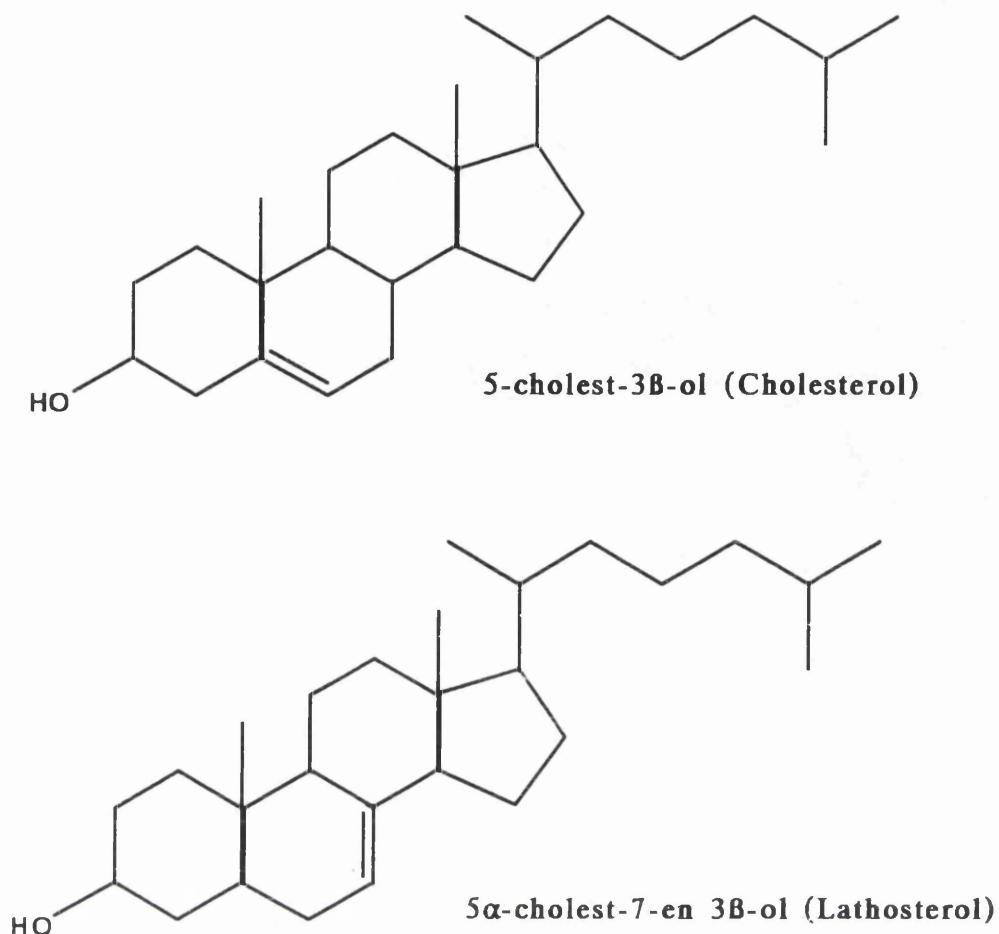
# Mev Run 5



**Figure 21.** Example of Standard Curve used in Mevalonic Acid Assay

## 2.7 Lathosterol

Levels of plasma 7-lathosterol ( $5\alpha$ -cholest-7-en- $3\beta$ -ol) have also been shown to correlate well with HMG CoA reductase activity in human liver (Bjorkhem *et al* 1987). This cholesterol precursor is not present in urine but may be assayed in plasma using a commercial derivatisation procedure followed by capillary gas chromatography. As shown in figure 22 lathosterol is an isomer of cholesterol differing only in the position of a single double bond. As such, it is difficult to separate these compounds by means other than an optimised Gas Liquid Chromatography system.



**Figure 22.** Structures of Cholesterol and Lathosterol with Systematic Names

### 2.7.1. Extraction and Derivatisation of Non-Saponifiable Lipids

The assay of lathosterol was performed by a modification of the procedure of Kempen *et al* (1988). A 200  $\mu$ L aliquot of plasma, taken at 08 00h after an overnight fast, was saponified using 1mL 0.3M NaOH in 90% ethanol incubated at 37°C for 60 min. After addition of 1mL distilled water, the non-saponifiable lipids were extracted with 4mL hexane and blown to dryness with nitrogen. 5 $\alpha$ -cholestane (10  $\mu$ g, Sigma) was added as an internal standard to each of samples. This compound was used because it is not normally present in human plasma. The non-saponifiable lipids were converted to their trimethylsilyl ethers using the commercial derivatisation preparation, Deriva-sil® (Chrompack Ltd, London UK) in which the active ingredients are BSTFA:TMCS:TSIM:Pyridine (3:2:3:10).

### 2.7.2. Gas Liquid Chromatography

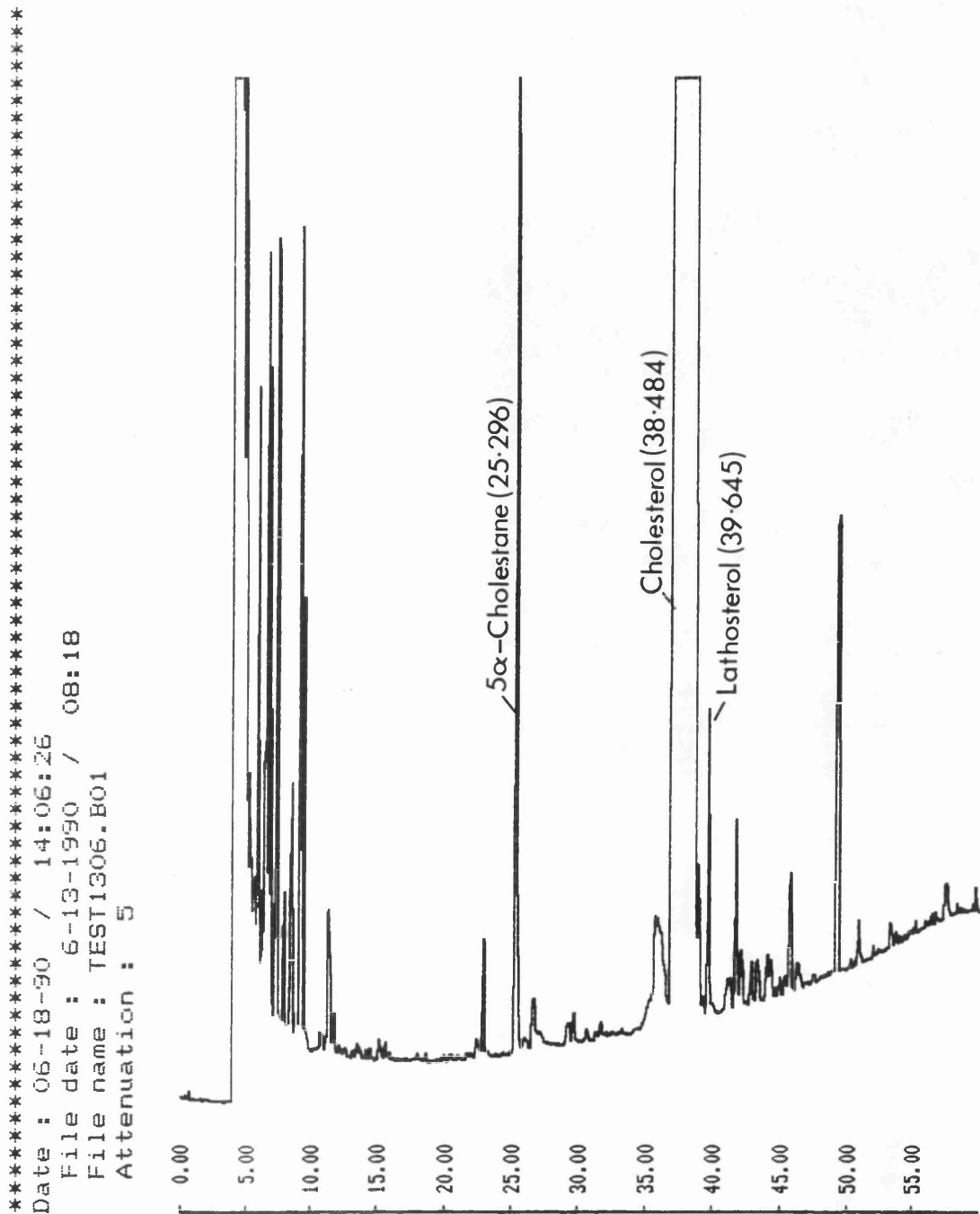
Lathosterol and cholesterol were quantified by capillary gas-liquid chromatography using the following conditions:

Chromatograph	CP9000 (Chrompack)
Column	WCOT 50m x 0.25 ID
Stationary Phase	CP-Sil 5CB Film Thickness 0.4 $\mu\text{m}$
Carrier Gas	Helium
Detection	Flame Ionisation
Temperature Program	Oven Initial 265°C Oven Final 320°C Rise 1°C. min <sup>-1</sup> for approximately 56 min
Injector Temperature	350°C
Detector Temperature	320°C

Examples of the tracings obtained from standards and from samples are shown in figure 23. The peaks obtained were integrated using the PCI integration software package (Chrompack) and quantified with reference to the internal standard 5 $\alpha$ -cholestane. Pure lathosterol and cholesterol were found to have relative retention times of 1.57 and 1.52 respectively in relation to the internal standard 5 $\alpha$ -cholestane. The procedure was found to have an inter-assay coefficient of variation of 10%.

### 2.7.3 Expression of Results

The final concentrations were expressed as lathosterol/cholesterol ratios. This practice was originally advocated by Miettinen (1970, 1982, 1985) as a way to correct for differences in the levels of cholesterol precursors that would occur as a mere consequence of a different number of acceptor (lipoprotein particles) in the circulation. Kempen *et al* (1988), continued this form of expression supported by their observations that in hypercholesterolaemic subjects both serum lathosterol and cholesterol levels are higher than normal controls, whereas the ratio is the same. This is in accord with the observation that cholesterol production rates are not elevated in hypercholesterolaemic subjects



**Figure 23.** Gas-liquid Chromatograph Pattern of Plasma Sterols. Cholesterol, 5 $\alpha$ -cholestane and lathosterol peaks are labelled with their retention times in minutes after injection. The rising baseline was a consistent feature in all lathosterol assays and is taken into account by the integration software. It is clear that the small lathosterol peak, although close to the much larger cholesterol peak (which appears blunted because of the scale of the graph), is distinct and separate.



as assessed by sterol balance studies (Grundy *et al* 1972). The increased serum lathosterol in hypercholesterolaemia may be due to the decreased clearance rate of its carrier, LDL. The effects of 'statin' therapy are correctly represented by a decrease in the lathosterol/cholesterol ratio. Kempen *et al* (1988) noted a 30% reduction in serum cholesterol but a 64% reduction in serum lathosterol so that the lathosterol/cholesterol ratio fell by 47%, reflecting enhanced clearance but also reduced synthesis of lathosterol.

## **2.8 Ethical Considerations**

All subjects gave informed written consent to the studies which met with the requirements of the Ethical Committee of each host institution viz. Glasgow Royal Infirmary, Hairmyres Hospital, East Kilbride and Victoria Infirmary, Glasgow. All injections of radioactive material were carried out under the ARSAC license of Professor James Shepherd.

## **2.9 Statistical Methods**

Differences between the lipid, lipoprotein and compositional data and metabolic parameters at different time points in the same individuals as presented in the results sections below were analysed by the paired students t-test. Because of the highly skewed distribution of plasma Lp(a), differences between the means of this analyte were assessed using the non-parametric Wilcoxon Sign Rank Test.

## Chapter 3    *Simvastatin*

...when you can measure what you are speaking about, and express it in numbers, you know something about it...when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts, advanced to the stage of Science...

Lord Kelvin.

### 3.1 Introduction

The publication of the findings from the Lipid Research Clinics Primary Prevention Trial (Lipid Research Clinics Program 1984a, 1984b) and more recently from the Helsinki Heart Study (Frick *et al* 1987) has confirmed the importance of lipid lowering as a means of preventing CHD. Since the completion of these studies, more powerful lipid regulating agents have become available; the most potent of these in terms of LDL cholesterol lowering are the 3-hydroxy, 3-methyl glutaryl coenzyme A (HMG CoA) reductase inhibitors or 'statins'. This is an important class of lipid lowering drugs whose remarkable efficacy has been documented in studies of several different groups of patients (Illingworth & Bacon 1989, Garg & Grundy 1989, Rabelink *et al* 1988, Vega & Grundy 1989b, Illingworth & O'Malley 1990, Vega, East & Grundy 1988, Mol *et al* 1988). The precise mechanism of action of these compounds, however, has not yet been elucidated fully. At first, it was thought that these drugs affected only LDL via activation of hepatic apoB/E receptors. However, further experience has suggested that they have substantial effects on very low (Sf 20-400), intermediate density (Sf 12-20), and high density lipoproteins (Grundy & Vega 1985, Vega, Krauss & Grundy 1990, Mol *et al* 1988) In general, all statins have weak but significant plasma triglyceride lowering

properties. While in type III hyperlipoproteinaemic subjects it has been shown that statins are both effective (Stuyt, Mol & Stalenhoef 1991) and can uniquely among lipid lowering drugs correct the compositional abnormality seen in VLDL (Vega *et al* 1988). Investigations of the kinetic changes underlying the LDL reduction on statin therapy has revealed an unsuspected heterogeneity of response. Many subjects, particularly those with familial hypercholesterolaemia (FH) exhibit an increase in apo-LDL clearance on drug (Bilheimer *et al* 1983). While in others decreased production of the lipoprotein is the principal cause of reduced LDL concentration in the circulation (Grundy & Vega 1985). This variable response to a drug with an apparently simple, known mechanism of action suggests that inhibition of cholesterol synthesis *in vivo* has a wide ranging effect on apolipoprotein B metabolism. Whether or not the statin induced changes are consequent on increased apoB/E receptor activity remains to be determined. Certainly in previous studies it has been demonstrated that the receptor has a role to play in the metabolism of apoB containing lipoproteins throughout most of the S<sub>f</sub> 0-400 density range (James *et al* 1989, Demant *et al* 1991a). Receptor deficiency in FH leads to accumulation of VLDL<sub>2</sub>, IDL, and LDL (James *et al* 1989). On the other hand the up-regulation of receptors in subjects with apoE2 homozygosity has a profound impact on all apoB containing classes (Demant *et al* 1991a).

The aim of the present study was to examine in detail the mechanism of action of HMG CoA reductase inhibitors in patients representative of the largest group who will receive such therapy, viz. those with primary, moderate hypercholesterolaemia. The dual VLDL<sub>1</sub> and VLDL<sub>2</sub> tracer turnover technique used has been previously applied to the study of the VLDL delipidation cascade in familial hypercholesterolaemia (James *et al* 1989), hepatic lipase deficiency (Demant *et al* 1988), lipoprotein lipase deficiency (Demant *et al* 1991b) and in subjects with different apoE phenotypes (Demant *et al* 1991a). While a new method to determine LDL substructure has been used successfully to examine the LDL subfraction distribution in normals and those with CHD (Krauss 1987b, Griffin *et al* 1990) and to look at the effects of other lipid lowering drugs (Griffin *et al* 1992), it was felt that application of these powerful techniques and correlation of kinetic parameters with VLDL and LDL structural information would yield new insight into lipoprotein physiology and the mechanism of action of the HMG CoA reductase inhibitor, simvastatin. This, indeed, proved to be the case. Significant correlations were observed between LDL apoB kinetic parameters and LDL substructure. The effects of the drug were isolated

to specific pathways throughout the delipidation cascade, which are believed to be receptor dependent.

### 3.2. Protocol

This was a single blind study comprising three phases designed to examine the influence of simvastatin on the metabolism of apoB containing particles. These phases were:

1. Preliminary Screening Period. During this 5 week run in period each patient was screened for cardiological, haematological, hepatic, endocrine, renal, ophthalmological and metabolic disease by routine clinical and laboratory testing.
2. Placebo Baseline Period. For 4 weeks each patient received placebo and in the final 2 weeks of this period a VLDL turnover study was performed on each patient to serve as a control. Baseline lipid, lipoprotein and lipoprotein subfractions were measured as described above.
3. Active Treatment Period. Immediately after the baseline assessment of apolipoprotein B metabolism the patients were commenced on simvastatin therapy at an initial dose of 10mg nocte rising to 20mg nocte after 4 weeks. Patients remained on this dose for 10 weeks. During the final 2 weeks each patient underwent a second turnover investigation while on simvastatin therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated.

### 3.3 Subjects

Eight study patients were selected from the Risk Factor Clinic at Glasgow Royal Infirmary and the Lipid Clinic at Hairmyres Hospital, East Kilbride. All patients had an initial elevated total cholesterol level ( $>7.0 \text{ mmol. L}^{-1}$ ) despite adherence to a standard lipid lowering diet (Study Group, European Atherosclerosis Society dietary recommendations, 1988) and a triglyceride level less than  $4.0 \text{ mmol. L}^{-1}$ , but did not have clinical evidence of FH. One subject's (SIM 02) cholesterol level fell below  $7.0 \text{ mmol. L}^{-1}$  but because he had previously satisfied the inclusion criteria on three occasions he was continued in the study. Another subject (SIM 04) completed the protocol but was subsequently found to have an E2/E2 phenotype. In view of recent findings by Demant *et al* (1991a) outlined above (3.1), regarding the effects of apo E2 homozygosity on apoB metabolism this subject was excluded from the

present analysis, which is based on the remaining 7 subjects. Patients were screened for cardiological, haematological, hepatic, endocrine, renal, ophthalmological and metabolic disease by routine clinical and laboratory testing. Pre-menopausal women were excluded from the study. The characteristics of each patient are summarised in Table 3. Five of the seven subjects studied were receiving prescribed medications for other clinical conditions, and these were continued unchanged throughout the course of the study.

### 3.4 Adverse Events

Simvastatin was well tolerated by all subjects participating in the study. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy. Similarly, ophthalmological examination with a slit lamp failed to detect alteration in the lens of any subject.

### 3.5 Lipids and Lipoproteins

Plasma lipid and lipoprotein levels of those patients studied are shown in Table 4. Treatment reduced plasma cholesterol by 29% ( $p < 0.001$ ). Triglyceride was not significantly affected although there was a tendency ( $p = 0.091$ ) for it to be decreased on therapy. The decrement in the former was due in this group to a 39% fall in LDL-cholesterol, while VLDL and HDL cholesterol were unchanged. Simvastatin therapy had no significant effect on plasma levels of Lp(a). Centrifugal analysis of the subfraction distribution in the LDL and HDL density intervals revealed that these had also been perturbed by simvastatin treatment. HDL<sub>2</sub> was increased significantly by 62% ( $p < 0.01$ ) while HDL<sub>3</sub> was unaffected. The change in HDL<sub>2</sub> concentration was correlated inversely with the fall in triglyceride for the group as a whole,  $r = 0.703$ . Figure 24 shows the LDL subfraction profile obtained upon density gradient fractionation of subjects' plasma. The pattern exhibited considerable variation from subject to subject but in most three distinct populations of particles could be distinguished both before and during therapy. The decrement in the major LDL-II species was the most obvious change in our subjects. Quantitative analysis (table 5) revealed that its concentration fell by 39% ( $p < 0.02$ ). LDL-I, the least dense fraction was also reduced significantly by 31% ( $p < 0.02$ ). Where LDL-III was high (figure 24, table 5) it appeared to fall substantially but a number of the subjects in whom it was a minor fraction recorded no change. Thus despite a

Table 3. Summary of Patient Characteristics

Subject	Sex†	Age§	BMI	Apo E Phenotype	Concomitant Drug Therapy	Clinical Diagnoses¶
SIM 01	M	47	25.5	4/4	Aspirin, Diltiazem, Atenolol	Angina
SIM 02	M	60	24.4	3/3	Aspirin, Temazepam	Angina, Hypertension, Osteoarthritis
SIM 03	M	36	26.0	3/2	Nil	Nil
SIM 05	M	57	24.5	4/3	Nil	Nil
SIM 06	M	48	24.4	3/3	Enalapril, Bumetanide, Potassium chloride	Angina, Hypertension
SIM 07	F	64	21.0	3/3	Amiloride, Hydrochlorothiazide, Atenolol	Angina, Hypertension
SIM 08	F	48	22.2	4/3	Atenolol	Angina, Osteoarthritis

†Male (M), Female (F). §Ages in years at start of study. || Body mass index (BMI) calculated from weight (kg) / height<sup>2</sup>(m). ¶Current medical problems of subjects at start of study.

Table 4. Lipid and Lipoprotein Changes with Simvastatin Therapy

Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	HDL <sub>2</sub> (mass)	HDL <sub>3</sub> (mass)	Lp(a)
-----mmol. L <sup>-1</sup> -----mg. dL <sup>-1</sup> -----mg. dL <sup>-1</sup> -----								
<i>Before Therapy</i>								
SIM 01A	7.5*	1.20	0.35	5.10	1.60	34	249	5
SIM 02A	6.5	1.00	0.40	4.75	1.35	47	256	4
SIM 03A	9.6	3.45	2.40	6.25	0.90	15	216	11
SIM 05A	7.2	2.25	0.75	5.00	1.40	42	257	30
SIM 06A	7.6	1.60	0.70	5.60	1.25	28	236	23
SIM 07A	7.4	1.75	0.75	5.25	1.40	53	240	103
SIM 08A	7.0	1.95	0.85	5.05	1.10	39	286	52
Mean (SEM)	7.5 (0.4)	1.89 (0.31)	0.90 (0.26)	5.29 (0.19)	1.29 (0.09)	39 (5)	249 (22)	33 (13)
<i>On Simvastatin</i>								
SIM 01B	5.0	1.20	0.60	2.95	1.45	90	211	6
SIM 02B	4.2	0.95	0.35	2.42	1.43	68	326	5
SIM 03B	6.3	2.15	1.40	4.25	0.90	17	194	16
SIM 05B	5.5	1.80	0.75	3.20	1.55	74	279	36
SIM 06B	6.0	1.20	0.55	4.10	1.30	61	285	37
SIM 07B	5.3	1.95	0.90	3.10	1.25	77	234	60
SIM 08B	4.6	1.25	0.60	2.75	1.20	51	306	45
Mean (SEM)	5.3 (0.3)	1.50 (0.17)	0.74 (0.13)	3.25 (0.26)	1.30 (0.08)	63 (24)	262 (50)	29 (8)
p	<0.001	NS	NS	<0.001	NS	<0.01	NS	NS

\* Cholesterol, triglyceride, VLDL, LDL & HDL-cholesterol figures are means of 3 measurements over the turnover.

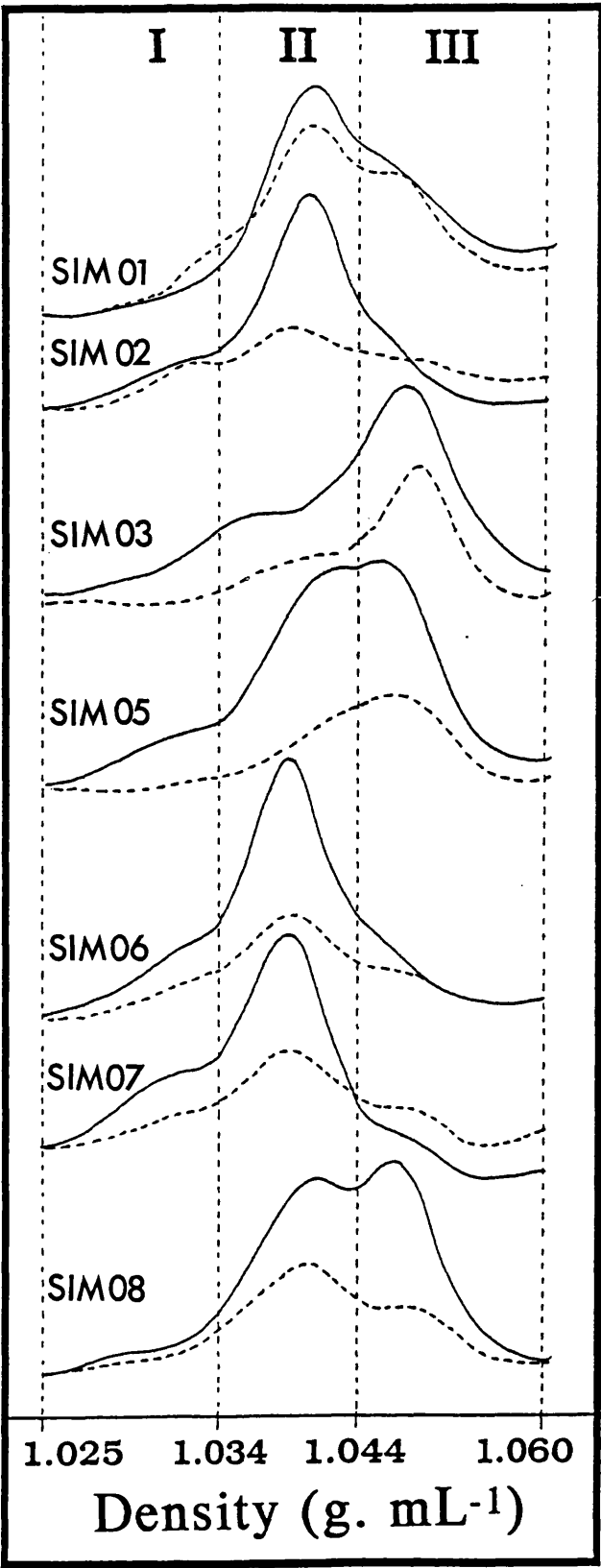
30% reduction in the mean value (table 5), the overall effect was not statistically significant. Compositional analysis of the four major apoB containing lipoprotein classes (table 6) demonstrated that the drug had profoundly altered the make-up of these species. A decrease in relative cholesteryl ester content was the most noticeable change in all fractions: in IDL, in particular, cholesteryl ester fell from 39.3% to 35.3%, while the triglyceride content rose from 11.5% to 18.6%. Simvastatin also decreased the percentage free cholesterol in IDL and LDL.

**Table 5.** LDL Subfraction Changes with Simvastatin Therapy.

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100ml plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
SIM 01A	640	66	392	182
SIM 02A	326	63	224	39
SIM 03A	358	39	136	183
SIM 05A	337	53	163	124
SIM 06A	378	70	255	53
SIM 07A	283	88	178	17
SIM 08A	329	30	175	124
Mean (SEM)	379 (44.9)	58 (7.4)	218 (32.7)	103 (25.6)
<i>On Simvastatin</i>				
SIM 01B	412	74	206	132
SIM 02B	185	48	102	35
SIM 03B	281	22	104	155
SIM 05B	224	16	141	67
SIM 06B	216	46	129	41
SIM 07B	224	54	137	33
SIM 08B	170	19	111	40
Mean (SEM)	245 (30.9)	40 (8.2)	133 (13.5)	72 (19.1)
p*	<0.001	<0.02	<0.02	NS
* Comparison of the means was performed using paired student's t-test.				





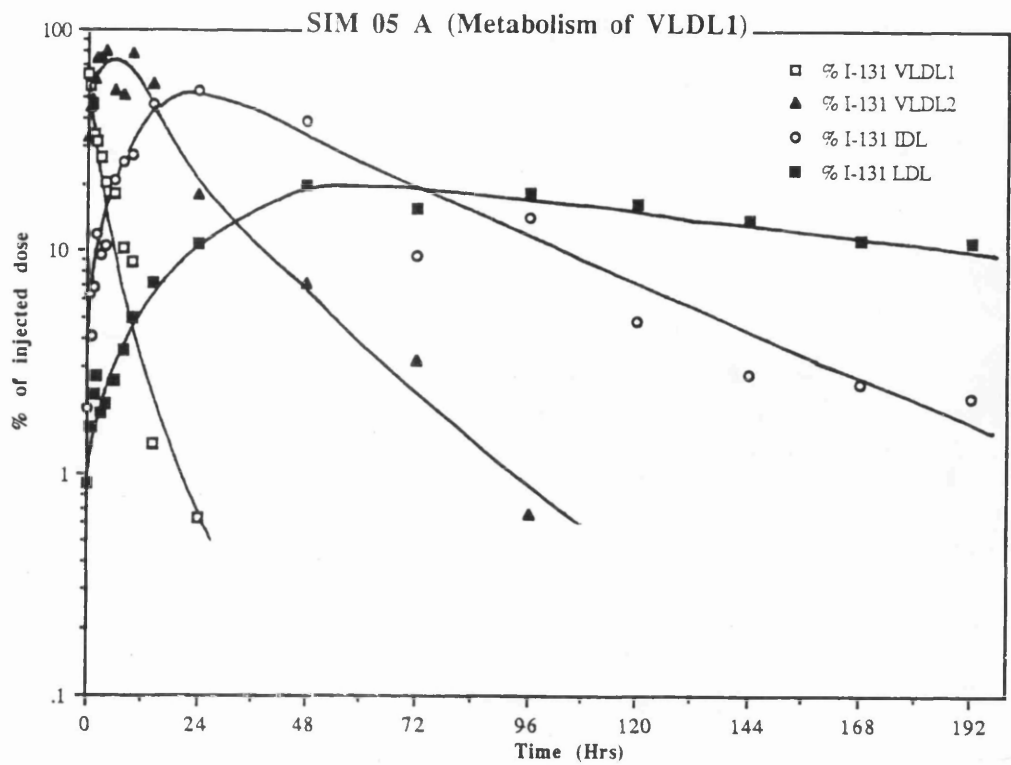


**Figure 24.** LDL subfraction profiles at baseline (—) and during simvastatin therapy (---) in each of the 7 subjects.

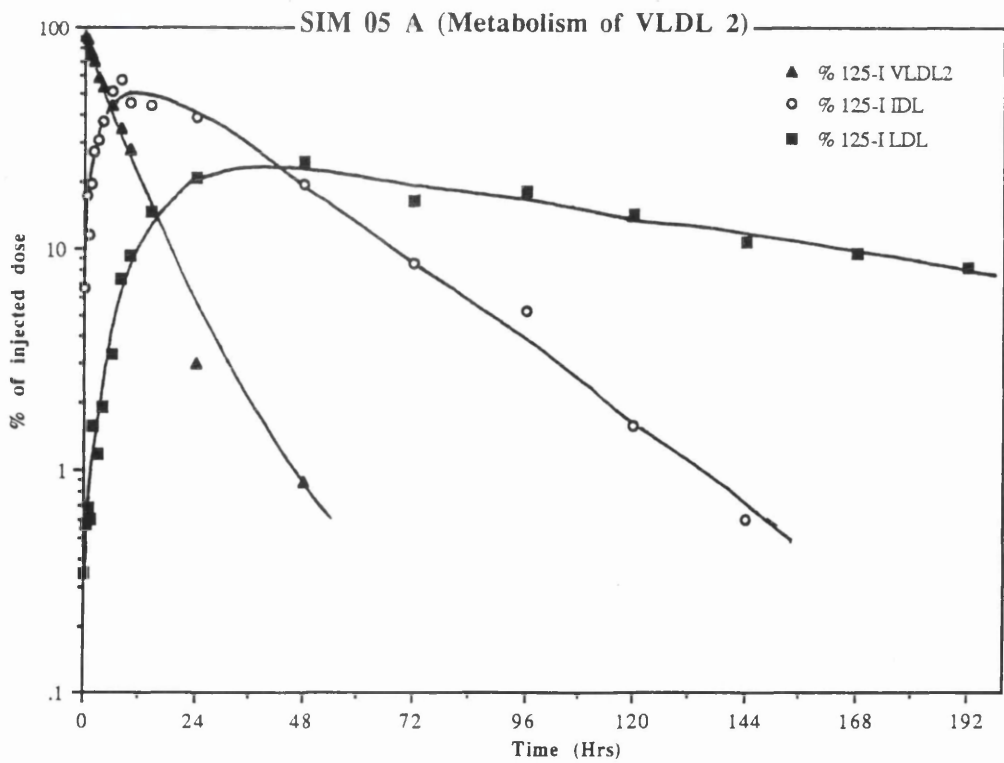
### 3.6 Apolipoprotein B Metabolism

The influence of simvastatin therapy on apolipoprotein B metabolism was examined before and on drug (representative decay curves for one subject, SIM 05 are shown in figures 25-28). ApoB disappeared rapidly from the VLDL<sub>1</sub> flotation interval and appeared virtually quantitatively in VLDL<sub>2</sub> over a period of 12-24 h after injection (figure 25). The decay of radioactive apoB in VLDL<sub>2</sub> was slower than in VLDL<sub>1</sub>, taking up to five days to reduce to 1% of the injected dose (figure 26). Drug therapy did not appear to affect the clearance rate of either VLDL<sub>1</sub> apoB (figure 27) or VLDL<sub>2</sub> apoB (figure 28). Similarly the extent of transfer of radioactive apoB into IDL was comparable before and during simvastatin therapy for both tracers. However, the decay rate of IDL apoB was faster on drug and the peak value attained in the LDL fraction consistently less than at baseline. In figure 26, LDL apoB radioactivity reaches a maximum of 25% of injected dose about 24 h after VLDL<sub>2</sub> injection whereas in figure 28, the LDL apoB maximum is only 13%. This finding indicates that the metabolic fate of IDL apoB is markedly affected by therapy with more material being lost directly from the circulation rather than being converted to LDL. In this subject (SIM 05), the clearance rate of LDL apoB radioactivity was also increased during simvastatin therapy. Kinetic rate constants and apoB fluxes were derived by compartmental modelling. The results for all 7 subjects are summarised in table 7; decay curves for the other six subjects and individual kinetic constants and masses are given are shown in appendix 2.

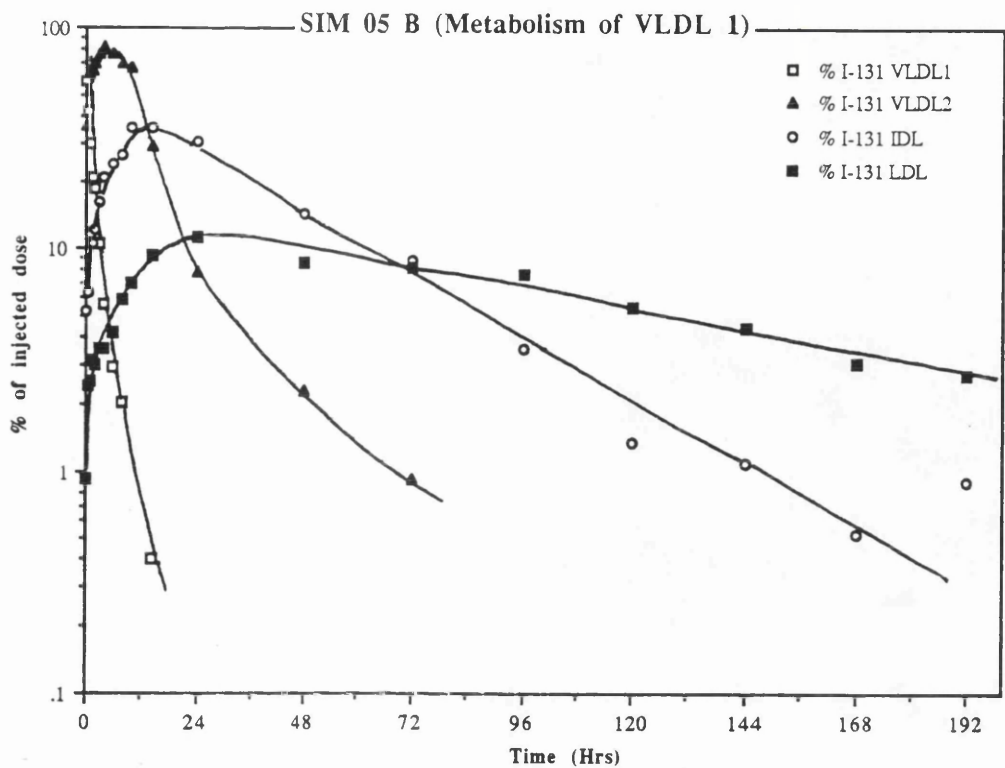
VLDL<sub>1</sub> apoB synthesis, pool size, and fractional transfer rate to VLDL<sub>2</sub> were unchanged by therapy and although the mean direct fractional catabolic rate of VLDL<sub>1</sub> apoB increased by some 209% from 1.7 to 5.3 pools. d<sup>-1</sup> this just failed to reach statistical significance ( $p=0.06$ ). The VLDL<sub>2</sub> apoB pool size remained unchanged on drug as did the amount of material derived from VLDL<sub>1</sub> or by direct input. There was, however, a rise in the mean VLDL<sub>2</sub> apoB direct fractional catabolic rate of 79%, from 1.69 to 3.02 pools.d<sup>-1</sup>. IDL formation from VLDL<sub>2</sub>, presumably by delipidation was the same before and during simvastatin therapy. The IDL apoB plasma pool size fell significantly from 1043 to 762mg (27%,  $p<0.01$ ) due entirely to an increase in direct catabolism (147%,  $p<0.05$ ) of this fraction. In contrast the IDL to LDL fractional transfer rate was not affected by the drug. Direct synthesis of LDL apoB decreased in 4 of the 7 subjects and the LDL apoB synthetic rate from VLDL<sub>2</sub>, and IDL showed a similar pattern with 5 of the 7 subjects showing a fall. The LDL



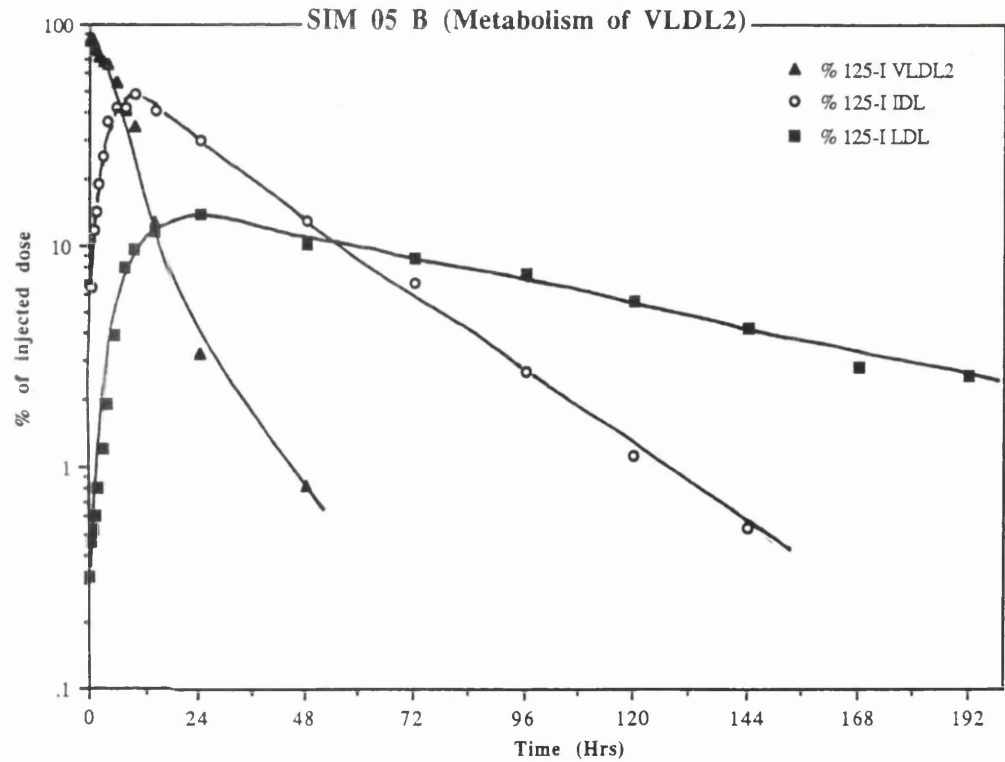
**Figure 25.** Baseline. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject SIM 05 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure 26.** Baseline Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject SIM 05 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure 27.** On Simvastatin. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject SIM 05 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>



**Figure 28.** On Simvastatin. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject SIM 05 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

**Table 7a.** VLDL<sub>1</sub> Apolipoprotein B Metabolism Before and On Simvastatin.

	Synthesis (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To VLDL <sub>2</sub> (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
SIM 01A	2280	215	5.26	5.3
SIM 02A	451	62	0.40	6.87
SIM 03A	1333	344	0.51	3.37
SIM 05A	517	126	0.82	3.29
SIM 06A	1491	109	0.03	13.65
SIM 07A	565	89	0.01	6.33
SIM 08A	716	68	5.07	4.67
Mean (SEM)	1050 (257)	145 (39)	1.73 (0.89)	6.22 (1.34)
<i>On Simvastatin</i>				
SIM 01B	3085	160	13.37	5.91
SIM 02B	1332	100	8.96	4.35
SIM 03B	856	154	1.76	3.80
SIM 05B	776	76	0.10	10.11
SIM 06B	3473	160	7.31	14.4
SIM 07B	456	77	0.01	5.91
SIM 08B	495	43	5.94	5.57
Mean (SEM)	1496 (475)	110 (18)	5.35 (1.89)	7.15 (1.43)
p *	NS	NS	NS	NS
*Differences between the means were assessed by the paired student's t-test.				

Table 7b. VLDL<sub>2</sub> Apolipoprotein B Metabolism Before and On Simvastatin.

	Direct Synthesis (mg.d <sup>-1</sup> )	Flux from VLDL <sub>1</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux to IDL+LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>					
SIM 01A	3065	1149	560	5.46	2.04
SIM 02A	1008	426	304	2.39	2.31
SIM 03A	1547	1158	1353	0.57	1.43
SIM 05A	1030	414	468	1.00	2.08
SIM 06A	155	1488	394	0.01	4.21
SIM 07A	974	564	517	1.49	1.51
SIM 08A	825	371	296	0.89	3.16
Mean (SEM)	1229 (343)	796 (173)	556 (138)	1.69 (0.69)	2.39 (0.37)
<i>On Simvastatin</i>					
SIM 01B	4088	946	429	9.13	2.53
SIM 02B	2867	436	364	3.59	5.42
SIM 03B	1754	585	718	1.12	2.14
SIM 05B	596	768	538	0.19	2.33
SIM 06B	678	2304	612	3.57	1.00
SIM 07B	1132	455	418	1.01	2.81
SIM 08B	1990	239	376	2.54	3.39
Mean (SEM)	1872 (476)	819 (263)	494 (50)	3.02 (1.13)	2.80 (0.52)
p	NS	NS	NS	NS	NS

**Table 7c.** IDL Apolipoprotein B Metabolism Before and On Simvastatin.

	Flux from VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
SIM 01A	1148	714	0.28	1.33
SIM 02A	700	678	0.45	0.58
SIM 03A	1926	1609	0.63	0.56
SIM 05A	971	983	0.60	0.39
SIM 06A	1666	1249	0.83	0.50
SIM 07A	769	1057	0.37	0.35
SIM 08A	933	1011	0.66	0.26
Mean (SEM)	1159 (176)	1043 (120)	0.55 (0.07)	0.57 (0.13)
<i>On Simvastatin</i>				
SIM 01B	1087	515	1.26	0.85
SIM 02B	1974	426	2.74	1.90
SIM 03B	1532	994	1.20	0.34
SIM 05B	1258	755	1.25	0.41
SIM 06B	61	1062	0.50	0.08
SIM 07B	1170	886	1.15	0.17
SIM 08B	1276	695	1.42	0.42
Mean (SEM)	1273 (158)	762 (90)	1.36 (0.26)	0.60 (0.24)
p	NS	<0.01	<0.05	NS



**Table 7d.** LDL Apolipoprotein B Metabolism Before and On Simvastatin.

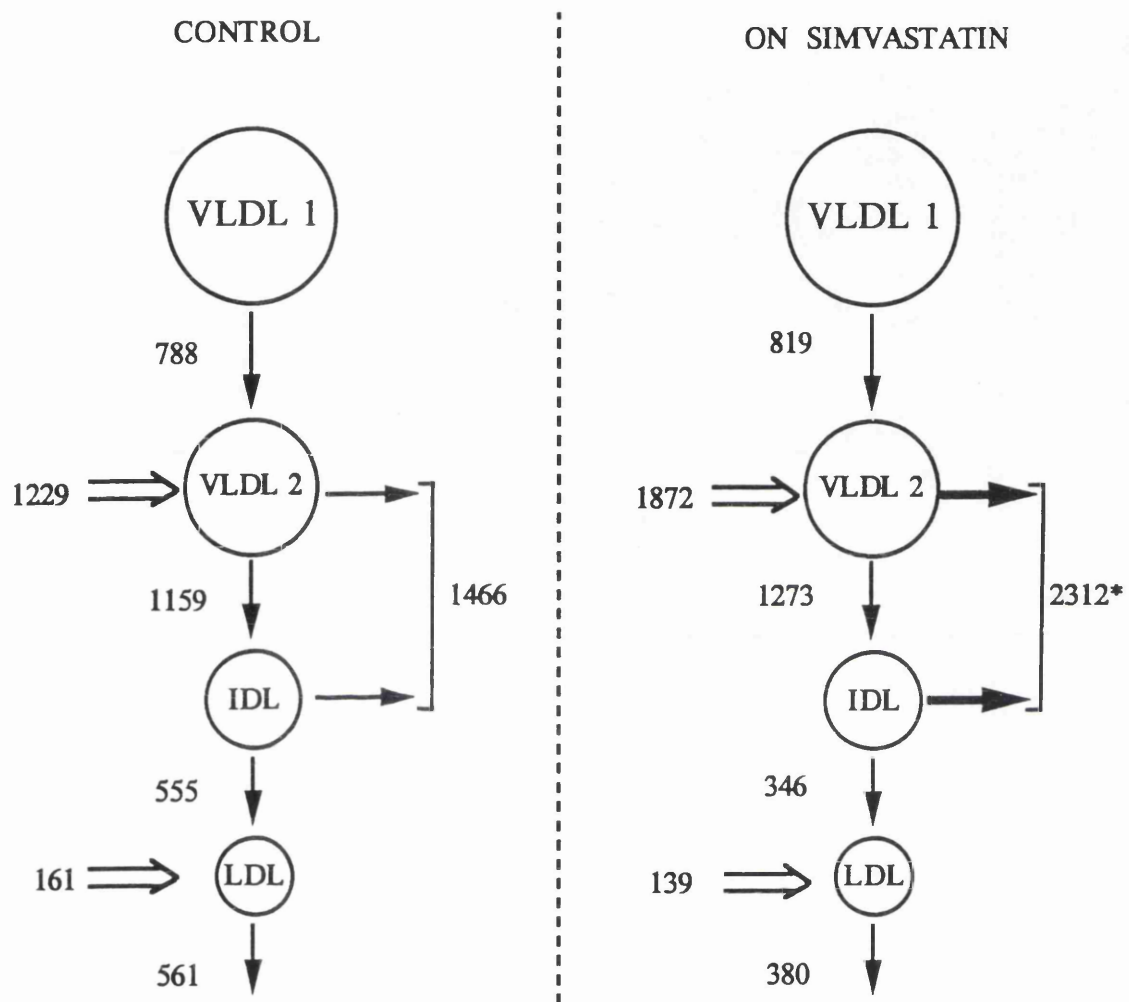
	Direct Synthesis ‡ (mg.d <sup>-1</sup> )	Flux from IDL + VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	VLDL-derived Plasma Pool (mg)	Fractional Catabolic Rate (pools.d <sup>-1</sup> )	TotalApoB Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>					
SIM 01A	385	950	2910	0.33	5730
SIM 02A	208	397	1203	0.33	1667
SIM 03A	77	910	3592	0.25	2957
SIM 05A	106	386	2160	0.18	1653
SIM 06A	103	641	2866	0.22	1749
SIM 07A	160	378	1918	0.20	1699
SIM 08A	86	266	1418	0.19	1627
Mean (SEM)	161 (41)	561 (104)	2295 (328)	0.24 (0.02)	2440 (778)
<i>On Simvastatin</i>					
SIM 01B	300	474	1837	0.26	7473
SIM 02B	81	825	1296	0.64	4280
SIM 03B	52	342	1406	0.24	2662
SIM 05B	123	315	1114	0.28	1495
SIM 06B	94	260	978	0.27	4245
SIM 07B	168	150	733	0.21	1756
SIM 08B	157	291	869	0.33	2642
Mean (SEM)	139 (31)	380 (83)	1176 (141)	0.32 (0.06)	3508 (577)
p	NS	NS	<0.01	NS	NS
‡	Direct synthesis in LDL was calculated as the difference between the total absolute catabolic rate (observed mass x overall FCR) and the input from VLDL and IDL.				

therapy, of 49% from 2295 to 1176mg ( $p<0.01$ ) due to a combination of reduced synthetic and increased catabolic rates. The mean fractional catabolic rate of LDL apoB, which we expected to increase in response to therapy did indeed rise by 33% but because of the scatter of responses this did not achieve overall significance. In this limited group of subjects it was the reduction in LDL apoB synthesis rather than any change in catabolism that appeared to be the predominant factor in reducing LDL. Before therapy 30% of apoB entering the delipidation cascade in these hyperlipidaemic subjects was converted to LDL. Following therapy the input remained the same but direct catabolism from VLDL<sub>2</sub> and IDL density ranges was increased significantly ( $p<0.05$ ) so that only 16% was processed to LDL (figure 29). It was also of note that the mean calculated total apoB synthetic rate increased from 2440 to 3508 mg/day ( $p=0.06$ ) in response to therapy (table 7).

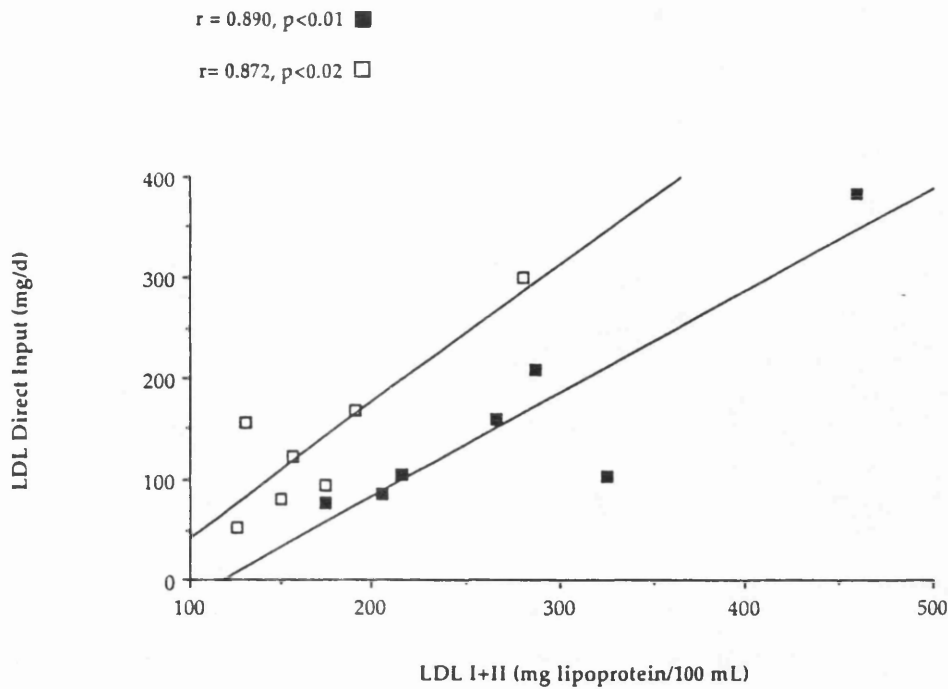
From these data, simvastatin is clearly responsible for greater effects on lipoprotein metabolism than a simple increase in LDL catabolism. In an attempt to correlate the changes in LDL kinetics with changes in LDL substructure, the relationship between the LDL apoB synthetic parameters and the plasma concentrations of LDL-I, II, and III was examined. LDL-I and II were grouped together for this analysis since they appeared to behave in a similar fashion, distinct from that of LDL-III. The relationship between LDL-I+II and LDL direct input and LDL-III and LDL direct input are shown in figures 30 and 31. The strong correlation between LDL-I+II and LDL direct input before ( $r=0.890$ ,  $p<0.01$ ) and after therapy ( $r=0.872$ ,  $p<0.02$ ) and the lack of correlation between LDL-III and LDL direct input was unexpected. The relationship between LDL-III and LDL synthesis from VLDL<sub>2</sub>, and IDL was also examined. Before therapy the correlation coefficient was  $r=0.633$ ,  $p=0.127$ : on therapy it was  $r=0.028$ ,  $p=0.952$ .

### 3.7 Discussion

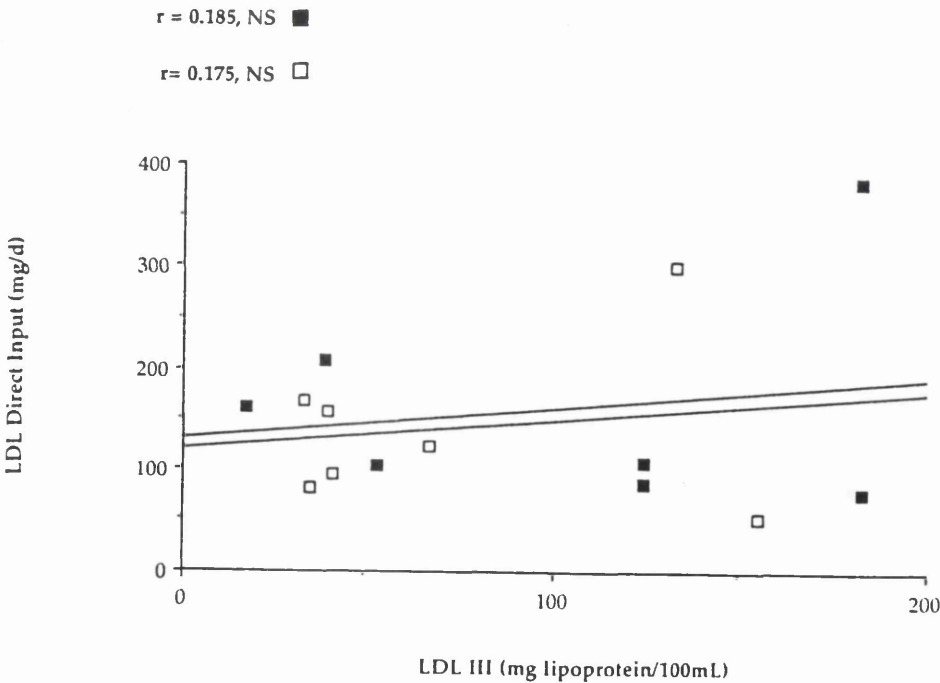
The mechanism by which the HMG CoA reductase inhibitors (statins) achieve their principal effect of lowering LDL cholesterol has been a particular focus of research over the last ten years. In an early study Bilheimer *et al* (1983) examined LDL turnover in familial hypercholesterolaemic subjects treated with mevinolin (lovastatin). They attributed the drug's LDL lowering to an increase in the apo-LDL fractional catabolic rate (FCR), which was thought to be secondary to the effect of the drug in suppressing cholesterol synthesis, reducing intracellular cholesterol concentrations and consequently promoting the



**Figure 29.** Summary of the effects of simvastatin therapy on apoB metabolism. Numbers on arrows represent mass transfer of apoB in mg.d<sup>-1</sup>. \* significantly different from control



**Figure 30.** Correlation between LDL subfractions I+II and LDL direct input at baseline (■) and during simvastatin therapy (□)



**Figure 31.** Correlation between LDL-III and LDL direct input at baseline (■) and during simvastatin therapy (□)

activity of the apoB/E receptor. A close parallel was drawn between the mechanism of action of the statins and that reported previously for bile acid sequestrant resins, which also showed an increase in receptor mediated LDL catabolism. No overall change in the apo-LDL synthesis rate was seen in these statin treated FH subjects although it was noted that 5 out of 6 examined showed reductions of up to 22% in this parameter. Further studies in patients with 'polygenic hypercholesterolaemia' (not attributable to FH) emphasised this dual action. In these studies (Grundy & Vega 1985, Vega & Grundy 1987) it was reported that both decreased output and increased clearance had to be invoked to explain the reduction in LDL in such patients. These workers proceeded to investigate apoB metabolism in VLDL, IDL and LDL and found that pravastatin therapy altered VLDL production but not the catabolism of any of these lipoprotein classes (Vega, Krauss & Grundy 1990). A summary of the findings of committed studies so far with this group of drugs is:

- i) that functional LDL receptors are essential for their action: FH homozygotes show no response to the drug in terms of altered plasma LDL levels or turnover (Uauy *et al* 1988),
- ii) subjects with a pronounced catabolic defect ie FH heterozygotes show an increase in LDL FCR on statins, and
- iii) other hypercholesterolaemic patients are as likely to demonstrate reduced apoB input into LDL as increased clearance of the lipoprotein, although, all subjects show a consistent 35-40% reduction in LDL pools however this is achieved.

The heterogeneous changes in the moderately hypercholesterolaemic group of subjects indicate perhaps an as yet unappreciated, underlying variability in apoB metabolism in the population. This group of subjects will, of course, be the largest group to receive these drugs.

Previously, the usefulness of dual tracer (VLDL<sub>1</sub> and VLDL<sub>2</sub>) studies has been demonstrated in delineating the role of receptors and lipases in the regulation of the VLDL to LDL delipidation cascade. Investigations in receptor defective FH homozygotes (James *et al* 1989) and normolipidaemic subjects who have defective apoE ligands (Demant *et al* 1991a) have revealed multiple steps where apoB/E receptors are involved in this metabolic pathway. If activation of receptors is the single most important action of the statins then this should be evident throughout the cascade; the results reported here indicate that this is the case. Simvastatin clearly stimulated lipoprotein catabolism at various stages in

the VLDL to LDL conversion and a number of these kinetic changes were linked to perturbations in the structure and composition of apoB containing lipoproteins. The drug's influence was not restricted to VLDL and LDL in our study. HDL<sub>2</sub>, the minor HDL subfraction, which is believed to have cardioprotective properties was increased by 62%. This change was apparently linked to the fall in total plasma triglyceride levels but could not be related to a perturbation in the kinetic behaviour of any particular VLDL subfraction.

The major finding of the present study was that simvastatin stimulated direct catabolism of lipoproteins throughout the VLDL delipidation cascade presumably as a result of its ability to increase apoB/E receptor activity. Certainly the pathways activated by the drug are those identified from previous turnover studies thought to involve receptor-mediated catabolism (James *et al* 1989). Lipolysis as measured by the fractional transfer rate of VLDL<sub>1</sub> to VLDL<sub>2</sub> and then on to IDL and LDL was not affected by therapy.

Since it is believed that apoB/E receptors play a major role in lipoprotein metabolism towards the denser end of the Sf 0-400 spectrum it is not surprising that IDL and LDL were the fractions most affected by therapy. There was not only a reduction in the plasma concentration, but a change in their composition. On therapy, there was a tendency for the cholesteryl ester content of all the apoB containing fractions to fall. This was most clearly seen in IDL where the absolute cholesteryl ester content of the particle decreased approximately 40%. These changes could have been due to either the synthesis of lipoproteins of altered composition, or perturbations of cholesteryl ester transfer (CETP) activity, or the selective removal of cholesteryl ester rich subpopulations from all of these lipoproteins. Bagdade *et al* (1990) have reported that statins do not appear to affect CETP and on the basis of the kinetic changes noted in the present study the last hypothesis, that increased receptor activity causes the selective removal of cholesteryl ester rich remnant lipoproteins, is favoured. There is *in vitro* evidence from cell culture studies that cholesteryl ester-rich remnant lipoproteins have a higher affinity for receptors than their triglyceride-rich counterparts (Koo *et al* 1988). Multicompartmental modelling showed that within the IDL subsystem it was the remnant pool (compartment 9, appendix 2) that appeared to be most influenced by simvastatin therapy. This interpretation is also in accord with the findings that statins uniquely among lipid lowering drugs decrease the VLDL remnant population in type III hyperlipoproteinaemia (Vega *et al* 1988). They alone are able to decrease the cholesteryl ester/

triglyceride ratio within this lipoprotein fraction towards normality. It is noteworthy that IDL appeared to be the most affected of all the apoB containing lipoproteins both in terms of its altered composition and its metabolic fate. This is significant in terms of the drug's mechanism of action since IDL possibly has the highest affinity of all the lipoprotein fractions to bind to the LDL receptor because it contains both apoB and the ancillary ligand apo E. This statin induced effect on IDL may also be clinically important because this species is quoted as a particularly atherogenic lipoprotein (Krauss 1987b).

These findings are at variance with the study of Vega *et al* (1990) who found that the catabolism of VLDL, IDL and LDL were not affected by pravastatin therapy. Rather, VLDL apoB synthesis was diminished in their patients during drug treatment. The reasons for this discrepancy between the two reports are not clear. It is doubtful that simvastatin and pravastatin have separate actions and likely that the inter-individual variation in response to the drugs seen in both studies led us to different conclusions as to the overall effect of the drugs. The patients in the study of Vega *et al* (1990) showed, like those presented here, no significant reduction in plasma triglyceride levels. However, they did show a diminution in VLDL cholesterol levels, whereas in the patients studied here VLDL cholesterol levels were not significantly affected by drug therapy. In view of the known metabolic effects of the statins the favoured idea is that in the majority of subjects their main action is to promote the removal of apoB/E containing lipoproteins from the delipidation cascade by direct catabolism.

As noted above increased catabolism alone did not explain the dramatic statin induced fall in LDL levels in moderate hypercholesterolaemic subjects. Decreased apoB transport down the delipidation cascade leading to diminished LDL production was equally important (figure 29). In this study, the opportunity was taken to examine the relationship between apoB kinetics and LDL subfraction distribution as defined by a recently developed density gradient technique (Griffin *et al* 1990). As in most subjects these patients demonstrated the presence of three LDL subfractions differing in size and density both before and during simvastatin therapy. In some subjects LDL-II was the predominant species while in others, small, dense LDL-III was the major form found. Overall the drug lowered LDL-I and LDL-II concentrations while the small, dense LDL-III were not significantly affected. These changes are to some extent pre-emptible and concordant with the idea that larger lipid-rich LDL are more easily removed by apoB/E receptors (Witztum *et al* 1985) and hence most

affected by increases in receptor activity. Surprisingly, however, a strong positive correlation was observed between the concentration of LDL-I and LDL-II combined and that computed as direct LDL synthesis both before and during simvastatin therapy. This kinetic parameter, which is calculated as the mass of LDL unaccounted for by VLDL to LDL catabolism, is the topic of considerable controversy (Shames & Havel 1991). The argument centres on whether the liver can elaborate LDL *per se* or as has been argued by Marzetta *et al* (1989) actually produces a rapidly metabolised LDL precursor. Whatever the route of apoB input into the LDL density range the pathway appears to be substantial in many patients, and accounts for the variation in LDL-I and LDL-II levels in our hypercholesterolaemic patients. It is not apparently affected by statin therapy as noted by the author (table 7) and Vega *et al* (1990), although this conclusion must be considered tentative since the kinetic parameters for LDL derived by direct synthesis are extrapolated from the behaviour of LDL, derived from VLDL.

An interpretation of the correlations between LDL kinetics and subfraction distribution, and the effects of statins is that in these moderately hypercholesterolaemic subjects LDL comes from two sources. One source is the classical VLDL to LDL delipidation cascade and it is this that is specifically affected by statin therapy. Statins indeed seem to have a very uniform effect on this pathway with most individuals showing approximately 50% reduction in the LDL that is associated with this route. The other pathway is highly variable in these subjects and is responsible for the variation in their levels of LDL-I and LDL-II. Clearly, more work needs to be done to delineate the properties of this pathway and to examine why it remains resistant to statin therapy. Effects of statins on the LDL subfraction profile must be compared with the effects of other lipid lowering agents such as nicotinic acid or its derivatives (Chapter 5) and fibrates (Chapter 6). Griffin *et al* (1992) have reported that triglyceride lowering drugs alter the distribution of LDL subfractions towards the less dense end LDL-I and LDL-II with a reduction in LDL-III. Statin therapy on the other hand appeared here to reduce LDL-I and LDL-II primarily as has been found for cholestyramine therapy (Griffin *et al* 1992). This differential effect of these drugs on LDL subfractions may have consequences for their potential anti-atherogenic properties. Further studies will elucidate the way in which LDL subfraction patterns are controlled and affected by drug therapy.



**In conclusion, statin therapy in moderate hypercholesterolaemia is associated with changes throughout the apoB containing lipoproteins and also in HDL. The changes are consistent with known mechanisms of action of the drug and result in alterations in both the composition and structure of lipoproteins.**

## Chapter 4 Colestipol

...a scientist must indeed be freely imaginative and yet sceptical, creative and yet a critic. There is a sense in which he must be free, but another in which his thought must be very precisely regimented; there is poetry in science, but also a lot of bookkeeping.

Peter B. Medawar, 1965.

### 4.1. Introduction

Interruption of the enterohepatic circulation and consequent hepatic depletion of the bile acid pool has been demonstrated by pharmacological and surgical means to be both effective as a lipid lowering strategy and as a preventive measure for CHD. The results from the LRC Coronary Primary Prevention Trial (Lipid Research Clinics Program 1984 a, 1984b) and more recently the St Thomas' Atherosclerosis Regression Study (Watts *et al* 1992) emphasise the clinical benefits of resin therapy in primary and secondary prevention, while Buchwald *et al* (1990) have convincingly demonstrated the benefits of surgical interruption of this pathway by partial ileal by-pass.

As surgical intervention is of limited clinical application drug therapy, using the bile acid sequestrant resins (or resins), has been widely used over a long period of time. These drugs have been used as lipid lowering agents for over 30 years (Bergen *et al* 1959). Two resins are currently available and, although they differ in structure (figure 8), they are considered to be clinically equivalent. Early studies by Parkinson, Gundersen & Nelson (1970) showed no difference between cholestyramine and colestipol in groups of hyperlipidaemic prisoners. Similarly, Glueck *et al* (1972) concluded after studying 25 patients in a crossover study that the two resins were, 'virtually indistinguishable'.

The mechanism of action of the resins and their pharmacology are described briefly in section 1.6.1 above. Resin therapy has been reported to reduce total plasma cholesterol by 18-25% (Grundy 1972, Glueck 1982, Levy *et al* 1984, Lipid Research Clinics Program 1984a) with average LDL-cholesterol reductions of 25-35% (Glueck 1982, Levy *et al* 1984, Lipid Research Clinics Program 1984a). Most studies also report small increases in plasma triglyceride (Glueck 1982, Levy *et al* 1984, Lipid Research Clinics Program 1984a). This change is usually not considered clinically relevant except in those patients with baseline hypertriglyceridaemia. Witztum *et al* (1979) have shown in their patients, however, this rise to be transient, persisting for only 2-10 weeks. Small increments in HDL-cholesterol have been reported (Levy *et al* 1984, Lipid Research Clinics Program 1984a) as have small but favourable increases in the HDL<sub>2</sub>/<sub>3</sub> ratio (Shepherd *et al* 1979b, Levy *et al* 1984).

The resins primarily exert these effects on the lipoprotein profile by preventing both the passive and active reabsorption of bile acids (Angelin 1992). The metabolic consequences of this are of considerable importance, affecting cholesterol and triglyceride synthesis and the activities of three key hepatic enzymes, cholesterol 7 $\alpha$  hydroxylase, phosphatidic acid phosphatase and HMG CoA reductase.

These drugs are worthy of further detailed metabolic study for two reasons. Firstly, the precise mode of cholesterol lowering and triglyceride raising remains to be fully elucidated, and secondly the important links between the cholesterol-lipoprotein and cholesterol-bile acid metabolic axes are not yet clearly defined (Einarsson & Angelin 1991).

The present study was not designed to answer all the remaining questions in this area but aimed to examine the effects of colestipol on the composition and metabolism of apoB containing lipoproteins VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL and LDL in a group of patients with moderate hypercholesterolaemia but with established coronary heart disease. The same dual tracer VLDL<sub>1</sub> and VLDL<sub>2</sub> turnover technique as used in chapter 3 was employed, as was the same ultracentrifugal analysis to examine the effects of colestipol therapy on LDL subfractions. Significant changes in lipoprotein composition were observed and are attributable to resin therapy. Multiple points in the delipidation cascade of apoB containing lipoproteins were also shown to be affected by therapy. The known

effects of this drug on expression of apoB/E receptors is invoked to explain these changes observed in the apoB kinetic parameters.

#### 4.2. Protocol

This study of the effects of colestipol on the metabolism of apoB containing particles consisted of three phases :

1. Preliminary Screening Period. During this 5 week run in period each patient was screened for cardiological, haematological, hepatic, endocrine, renal, ophthalmological and metabolic disease by routine clinical and laboratory testing.
2. Baseline Study. In this period a VLDL turnover study was performed on each patient to serve as a control. Baseline lipid, lipoprotein and lipoprotein subfractions were measured as described above.
3. Active Treatment Period. Immediately after the baseline assessment of apolipoprotein B metabolism the patients were commenced on colestipol therapy rising to a dose of 20g. day<sup>-1</sup> over a period of 7-10 days. Patients remained on this dose for 10 weeks. During the final 2 weeks each patient underwent a second turnover investigation while on colestipol therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated.

#### 4.3 Subjects

Eight study patients were selected from the Cardiac Surgery database at Glasgow Royal Infirmary. All patients were male and had an initial elevated total cholesterol level ( $\geq 6.5$  mmol. L<sup>-1</sup>) despite adherence to a standard lipid lowering diet (Study Group, European Atherosclerosis Society dietary recommendations, 1988) and a triglyceride level less than 3.0 mmol. L<sup>-1</sup>, but did not have clinical evidence of FH. All subjects had undergone coronary artery by-pass grafting between 3 and 12 months previously. Patients were screened for cardiological, haematological, hepatic, endocrine, renal and metabolic disease by routine clinical and laboratory testing to exclude secondary causes of hyperlipidaemia and any illness thought to influence the outcome of the study. The characteristics of each patient are summarised in table 8. All eight subjects studied were receiving prescribed medications as part of their post

Table 8. Summary of Patient Characteristics

Subject*	Sex†	Age§	BMI	Apo E Phenotype	Concomitant Drug Therapy	Clinical Diagnoses¶
COL 01	M	50	22.2	3/3	aspirin, dipyridamole, timolol (eye drops), naproxen	Glaucoma, osteoarthritis
COL 02	M	45	29.2	4/3	aspirin, dipyridamole, propanolol	Nil
COL 03	M	64	24.1	3/3	aspirin, dipyridamole, frusemide	Prostatism
COL 04	M	44	24.2	3/3	aspirin, dipyridamole	Nil
COL 05	M	53	26.0	3/3	aspirin, dipyridamole	Nil
COL 06	M	51	30.4	4/4	aspirin, dipyridamole, cimetidine	Peptic ulceration
COL 07	M	55	25.3	3/2	aspirin	Hypertension
COL 08	M	45	29.4	4/4	aspirin, dipyridamole	Nil

†Male (M). §Ages in years at start of study. || Body mass index (BMI) calculated from weight (kg) / height<sup>2</sup>(m). ¶Current medical problems of subjects at start of study in addition to coronary heart disease.

operative management, and four were receiving drugs for other clinical conditions. All prescribed medications were continued unchanged throughout the course of the study.

#### 4.4 Adverse Events

Colestipol was reasonably well tolerated by all subjects participating in the study. Two patients reported constipation and one patient dyspepsia, but in none of the three cases was this severe enough to necessitate intervention or withdrawal of drug. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy.

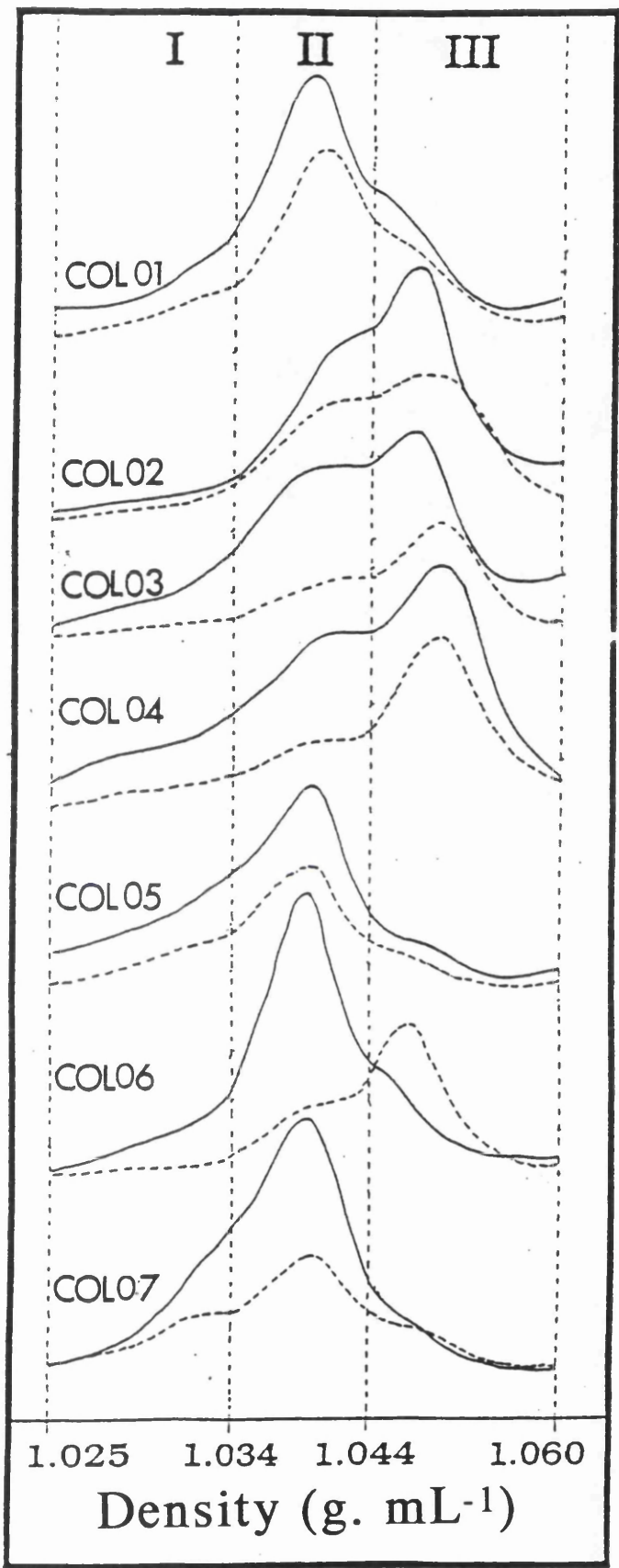
#### 4.5 Lipids and Lipoproteins

Plasma lipid and lipoprotein levels of those patients studied are shown in table 9. Treatment reduced plasma cholesterol by 14% ( $p < 0.002$ ). This decrement was due, in this group, to a 23% fall in LDL-cholesterol ( $p < 0.002$ ), while VLDL and HDL cholesterol were unchanged. There was also, as expected, a non-significant rise in triglyceride of 21%. Centrifugal analysis of the subfraction distribution in the HDL and LDL density intervals was performed. Due to analytical difficulty three HDL subfraction samples were lost and analysis was performed on the remaining five pairs of data. No significant change in HDL<sub>2</sub>, HDL<sub>3</sub> or the HDL<sub>2/3</sub> ratio was observed, although the incomplete nature of this data renders the sample size too small for reasonable comment. Figure 32 shows the LDL subfraction profiles obtained upon density gradient fractionation of subjects' plasma. There was some inter-individual variation in the pattern observed, but in most three distinct populations of particles could be distinguished both before and during therapy. Again, one data set for subject COL 08 on colestipol was lost during analysis and comparison of means is performed on the remaining 7 pairs of data. The decrement in the major LDL-II species was the most significant change in these subjects. Quantitative analysis revealed that its concentration fell by 38% ( $p < 0.005$ ). LDL-I, the least dense fraction was also reduced significantly by 38% ( $p < 0.01$ ). The colestipol induced response in LDL-III was variable (figure 32, table 10). In 3 individuals it appeared to fall substantially, but another 3 subjects recorded no change with one subject (COL 06) showing a marked increase. Thus despite a 23% reduction in the mean value (table 10), the overall effect was not statistically significant. Compositional analysis of the

Table 9. Lipid and Lipoprotein Changes with Colestipol Therapy

Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	HDL <sub>2</sub> (mass)	HDL <sub>3</sub> (mass)	Lp(a)
			mmol. L <sup>-1</sup>			mg. dL <sup>-1</sup>	mg. dL <sup>-1</sup>	mg. dL <sup>-1</sup>
<i>Before Therapy</i>								
COL 01A	5.82*	1.32	0.52	4.23	1.08	61	220	6
COL 02A	6.43	2.65	1.12	4.20	1.08	23	233	7
COL 03A	6.98	2.05	1.03	4.90	1.05	†	†	90
COL 04A	7.27	1.82	0.70	5.50	1.07	29	235	2
COL 05A	6.62	1.72	0.80	4.58	1.23	38	204	7
COL 06A	6.92	1.83	0.82	5.02	1.08	26	260	33
COL 07A	6.02	1.52	0.73	3.70	1.03	102	199	5
COL 08A	6.52	2.05	0.97	4.38	1.17	64	224	10
Mean (SEM)	6.57 (0.17)	1.87 (0.14)	0.84 (0.07)	4.56 (0.20)	1.10 (0.02)	52 (14) n=5	224 (11) n=5	20 (11)
<i>On Colestipol</i>								
COL 01B	5.35	1.85	0.57	3.48	1.30	†	†	5
COL 02B	5.55	2.37	0.97	3.62	0.97	†	†	†
COL 03B	5.55	1.95	0.88	3.60	1.07	51	196	62
COL 04B	6.40	2.69	1.20	4.15	1.05	39	210	13
COL 05B	5.43	1.53	0.72	3.40	1.32	119	239	4
COL 06B	5.45	2.33	1.08	3.28	1.08	38	275	22
COL 07B	5.60	1.33	0.77	3.48	1.42	72	224	12
COL 08B	6.13	4.03	1.93	3.07	1.13	26	250	8
Mean (SEM)	5.68 (0.13)	2.26 (0.30)	1.02 (0.15)	3.51 (0.11)	1.17 (0.06)	59 (17) n=5	240 (11) n=5	18 (8) n=7
p	<0.002	NS	NS	<0.002	NS	NS	NS	NS

\* Cholesterol, triglyceride, VLDL, LDL & HDL-cholesterol figures are means of 3 measurements over the turnover.  
† Sample lost during analysis



**Figure 32.** LDL subfraction profiles at baseline (—)and during colestipol therapy (---) in subjects COL 01-COL 07.



**Table 10.** LDL Subfraction Changes with Colestipol Therapy

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100mL plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
COL 01A	367	51	248	68
COL 02A	505	55	240	210
COL 03A	344	45	166	133
COL 04A	584	107	261	216
COL 05A	305	88	193	24
COL 06A	401	62	285	55
COL 07A	362	104	228	31
COL 08A	437	79	154	205
Mean	413	74	222	118
(SEM)	(32.6)	(8.5)	(16.4)	(29.5)
Mean (n=7)	410	73	232	105
(SEM)	(37.4)	(9.8)	(15.3)	(30.9)
<i>On Colestipol</i>				
COL 01B	220	38	136	46
COL 02B	258	41	114	103
COL 03B	225	25	98	102
COL 04B	322	41	169	112
COL 05B	300	72	202	26
COL 06B	302	24	135	143
COL 07B	258	71	153	34
COL 08B†	†	†	†	†
Mean (n=7)	269	45	144	81
(SEM)	(15.0)	(7.4)	(13.1)	(17.0)
p*	<0.01	<0.01	<0.005	NS

† Sample lost during analysis

\* Comparison of the means was performed using paired student's t-test.

four major apoB containing lipoprotein classes (table 11) demonstrated that colestipol had altered the make-up of these species. There appeared to be a differential effect of drug therapy on the relatively triglyceride-rich lipoproteins (viz. VLDL<sub>1</sub> and VLDL<sub>2</sub>) and the relatively cholesterol-rich species (viz. IDL and LDL). The former became larger with increased content of all components although only triglyceride and phospholipid enrichment achieved statistical significance in VLDL<sub>2</sub> ( $p < 0.05$  in both cases). IDL and LDL on the other hand appeared to become overall reduced in mass particularly in terms of free

**Table 11. Composition of Apolipoprotein B Containing Lipoproteins Before and On Colestipol Therapy.**

	Free Cholesterol	Cholesteryl Ester	Triglyceride (mg. dL <sup>-1</sup> )	Phospholipids	Apo B	
VLDL <sub>1</sub>	Before Therapy On Colestipol	3.44 (1.11) * 6.99 (2.22)	14.55 (2.61) 18.89 (3.04)	70.47 (12.54) 95.14 (18.22)	20.00 (2.40) 26.40 (5.67)	3.24 (0.56) 4.03 (0.59)
VLDL <sub>2</sub>	Before Therapy On Colestipol	4.24 (0.53) 5.98 (0.95)	16.03 (1.67) 21.03 (2.94)	24.00 (2.10) 34.66 (4.24) §	14.08 (0.90) 19.48 (2.45) §	3.45 (0.31) 3.42 (0.48)
IDL	Before Therapy On Colestipol	8.00 (1.95) 4.25 (0.73) §	30.35 (2.85) 26.50 (2.66)	11.69 (2.13) 9.77 (1.11)	19.41 (2.60) 18.01 (3.54)	6.03 (0.51) 4.72 (0.49) †
LDL	Before Therapy On Colestipol	42.58 (2.23) 24.63 (3.32) †	132.03 (4.53) 114.14 (17.95)	18.52 (1.82) 17.56 (2.39)	79.19 (3.28) 66.44 (8.01)	64.55 (8.93) 58.93 (6.64)

\* Mean (SEM)  
† Significantly different from before therapy, †p<0.001, ‡p<0.01, §p<0.05 by paired student's t-test

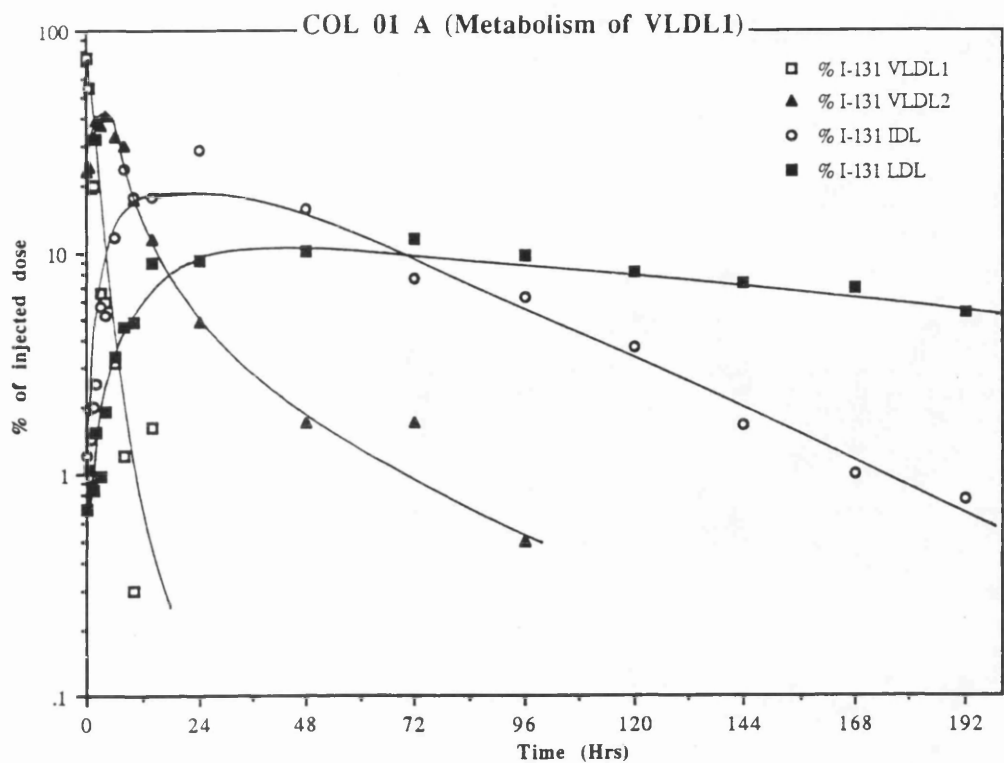
cholesterol (IDL by 47%,  $p < 0.05$ , LDL by 42%,  $p < 0.05$ ) and protein (IDL by 24%,  $p < 0.05$ , LDL by 32%, NS). There was also a marked fall in mean LDL-cholesterol:apoB ratio of 58%.

#### 4.6 Apolipoprotein B Metabolism

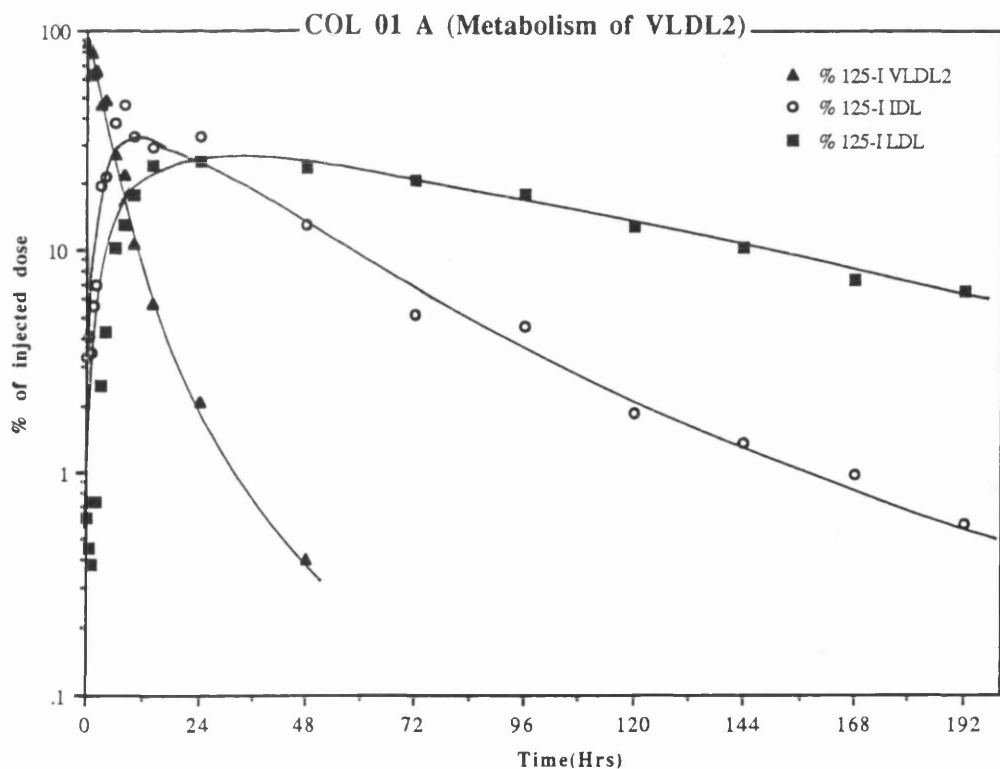
The influence of colestipol therapy on apolipoprotein B metabolism was examined before and on drug (representative decay curves for one subject, COL 01 are shown in figures 33-36). ApoB disappeared rapidly from the VLDL<sub>1</sub> flotation interval and appeared in VLDL<sub>2</sub> over a period of 6-18 h after injection (figure 33). The decay of radioactive apoB in VLDL<sub>2</sub> was slower than in VLDL<sub>1</sub>, taking almost 2 days to reduce to 1% of the injected dose (figure 34). Colestipol therapy appeared to increase the clearance rate of both VLDL<sub>1</sub> apoB (figures 35) and VLDL<sub>2</sub> apoB (figures 36). The flux of apoB into IDL was comparable before and during therapy for both tracers. However, the decay rate of IDL apoB appeared faster on drug with the peak value attained in the LDL interval being consistently less than at baseline. Before therapy, LDL apoB radioactivity reached a maximum of 25% of injected dose about 24 h after VLDL<sub>2</sub> injection (figure 34), whereas during therapy (figure 36) the LDL apoB maximum was only 15%. These findings suggest that the metabolic fate of IDL apoB is markedly affected by therapy with more material being lost directly from the circulation rather than being converted to LDL. In this subject (COL 01) the clearance rate of LDL apoB radioactivity appeared unchanged by colestipol therapy.

Kinetic rate constants and apoB fluxes were derived by compartmental modelling as described in chapter 2. The results for all 8 subjects are summarised in table 12; decay curves for the other 7 subjects and individual kinetic constants and masses are shown in appendix 3.

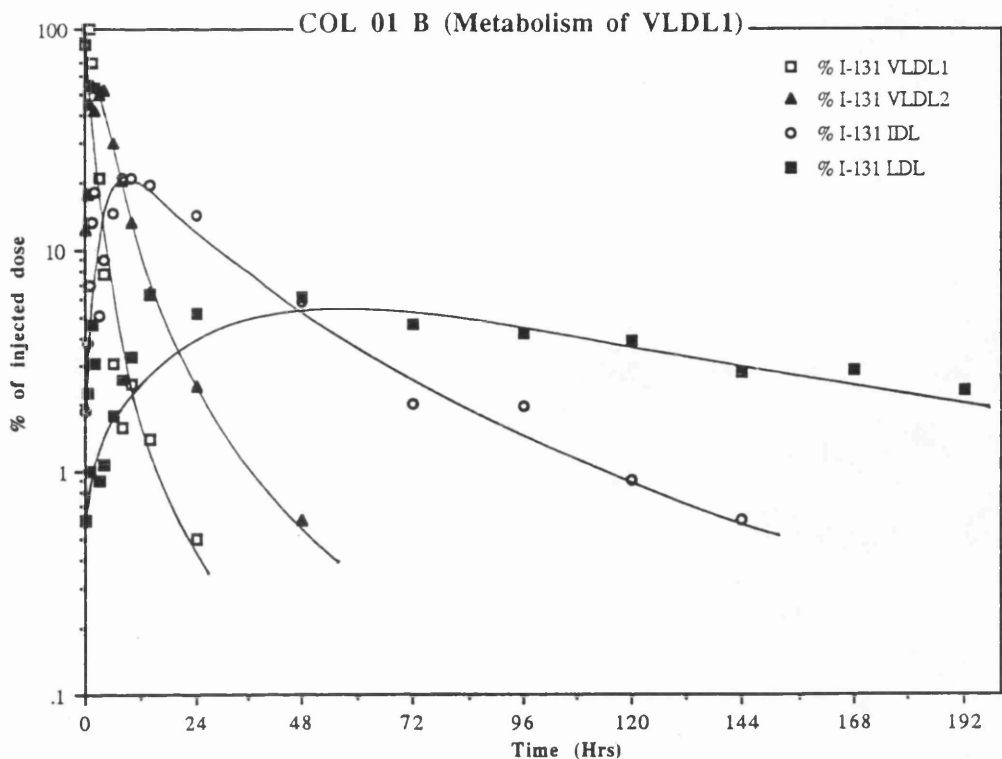
VLDL<sub>1</sub> apoB synthesis, pool size, and fractional transfer rate to VLDL<sub>2</sub> were unchanged by therapy but the mean direct fractional catabolic rate of VLDL<sub>1</sub> apoB was reduced by 49% ( $p < 0.05$ ) from 2.5 to 1.3 pools.  $d^{-1}$ . The VLDL<sub>2</sub> apoB pool size was expanded on drug therapy by 36% ( $p < 0.05$ ). Although no other change in VLDL<sub>2</sub> kinetic parameters achieved statistical significance this alteration in pool size was associated with increases in VLDL<sub>2</sub> apoB direct input in 6 out of 8 subjects and increased input from VLDL<sub>1</sub> in 7 out of 8. IDL formation from VLDL<sub>2</sub> was unaffected by resin therapy but there was a



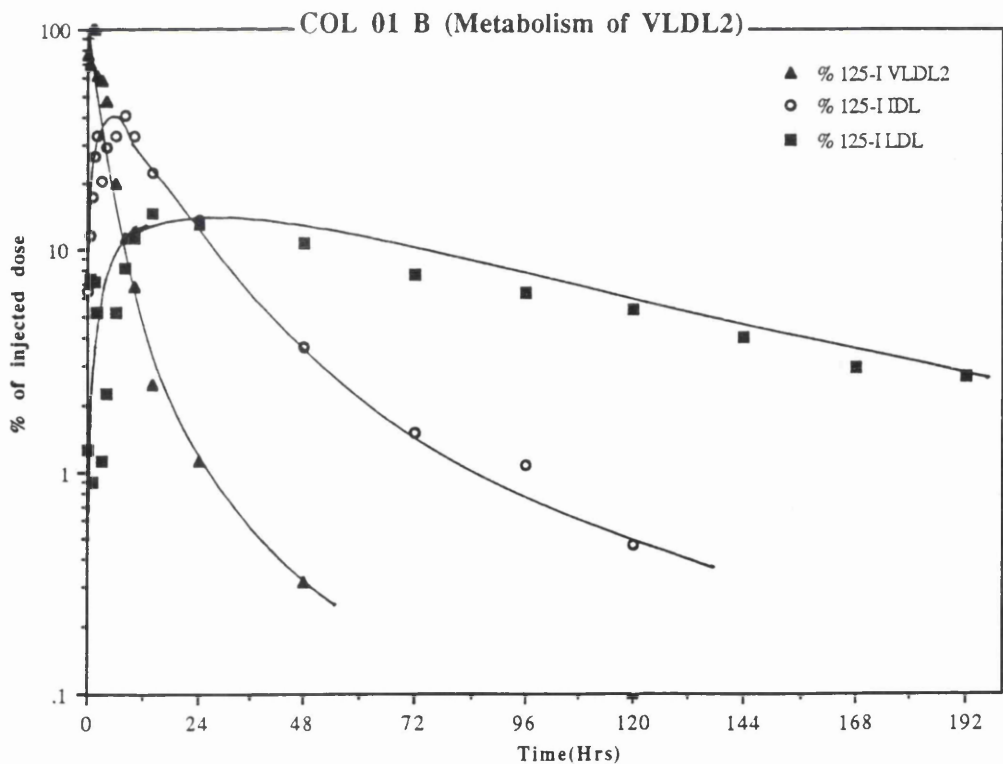
**Figure 33.** Baseline. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure 34.** Baseline Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure 35.** On Colestipol. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>



**Figure 36.** On Colestipol. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

Table 12a VLDL<sub>1</sub> Apolipoprotein B Metabolism before and on Colestipol

Subject	Synthesis (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To VLDL <sub>2</sub> (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
COL 01A	758	90	3.99	4.43
COL 02A	1863	242	3.23	4.46
COL 03A	541	42	0.01	12.87
COL 04A	354	41	0.01	8.62
COL 05A	510	71	3.38	3.79
COL 06A	1597	166	2.48	7.14
COL 07A	505	63	2.99	5.01
COL 08A	1187	182	4.17	2.35
Mean (SEM)	914 (200)	112 (26)	2.53 (0.58)	6.08 (1.19)
<i>On Colestipol</i>				
COL 01B	1061	123	3.48	5.14
COL 02B	1240	220	0.11	5.53
COL 03B	400	89	0.01	4.48
COL 04B	464	94	0.57	4.37
COL 05B	423	69	0.01	6.12
COL 06B	1310	240	0.37	5.09
COL 07B	253	55	1.97	2.64
COL 08B	2109	324	3.68	2.83
Mean (SEM)	907 (225)	151 (34)	1.28 (0.55)	4.53 (0.44)
p *	NS	NS	<0.05	NS
* Differences between the means were assessed by the paired student's t-test.				

Table 12b. VLDL<sub>2</sub> Apolipoprotein B Metabolism before and on Colestipol

	Direct Synthesis (mg.d <sup>-1</sup> )	Flux from VLDL <sub>1</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux to IDL+ LDL (pools.d <sup>-1</sup> )
<i>Before Therapy</i>					
COL 01A	484	399	287	1.47	1.61
COL 02A	311	1079	259	1.64	3.75
COL 03A	517	541	234	0.48	4.01
COL 04A	661	354	254	1.04	3.99
COL 05A	1111	269	263	1.68	3.57
COL 06A	289	1185	352	0.93	3.28
COL 07A	267	316	319	0.04	1.79
COL 08A	964	428	498	0.22	2.58
Mean (SEM)	576 (112)	534 (137)	308 (30)	0.94 (0.23)	3.07 (0.34)
<i>On Colestipol</i>					
COL 01B	631	632	262	1.38	3.43
COL 02B	339	1217	386	2.15	1.88
COL 03B	568	399	409	0.33	2.04
COL 04B	1534	410	622	1.75	1.38
COL 05B	705	423	257	1.79	2.60
COL 06B	716	1222	532	1.26	2.39
COL 07B	736	145	389	0.03	2.23
COL 08B	479	918	503	1.00	1.77
Mean (SEM)	714 (127)	671 (143)	420 (45)	1.21 (0.26)	2.22 (0.22)
p	NS	NS	<0.05	NS	NS

**Table 12c.** IDL Apolipoprotein B Metabolism before and on Colestipol.

	Flux from VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
COL 01A	434	573	0.12	0.64
COL 02A	958	650	0.45	1.02
COL 03A	881	656	0.40	0.95
COL 04A	988	1058	0.34	0.59
COL 05A	929	877	0.39	0.67
COL 06A	1076	900	0.88	0.88
COL 07A	566	700	0.20	0.61
COL 08A	1286	833	0.57	0.97
Mean (SEM)	890 (96)	781 (58)	0.42 (0.08)	0.79 (0.06)
<i>On Colestipol</i>				
COL 01B	893	546	1.13	0.51
COL 02B	703	505	0.32	1.08
COL 03B	760	526	0.71	0.73
COL 04B	849	922	0.24	0.68
COL 05B	661	599	0.35	0.76
COL 06B	1270	761	0.74	0.92
COL 07B	862	675	0.56	0.72
COL 08B	894	889	0.22	0.78
Mean (SEM)	862 (66)	678 (58)	0.53 (0.11)	0.77 (0.06)
p	NS	<0.03	NS	NS



**Table 12d.** LDL Apolipoprotein B Metabolism before and on Colestipol.

	Direct Synthesis ‡ (mg.d <sup>-1</sup> )	Flux from IDL + VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	VLDL-derived Plasma Pool (mg)	Fractional Catabolic Rate (pools.d <sup>-1</sup> )	Total ApoB Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>					
COL 01A	351	488	1532	0.32	1593
COL 02A	130	681	3191	0.21	2304
COL 03A	82	677	2331	0.29	1140
COL 04A	203	652	2431	0.27	1218
COL 05A	405	601	1921	0.31	2026
COL 06A	493	869	3271	0.27	2379
COL 07A	342	431	1305	0.33	1114
COL 08A	394	810	3284	0.25	2545
Mean (SEM)	300 (51)	651 (52)	2408 (279)	0.28 (0.01)	1790 (211)
<i>On Colestipol</i>					
COL 01B	197	282	1003	0.28	1889
COL 02B	291	563	1950	0.29	1870
COL 03B	43	460	1637	0.28	1011
COL 04B	0	636	2993	0.21	1998
COL 05B	56	464	2290	0.20	1184
COL 06B	103	703	3041	0.23	2129
COL 07B	0	492	2308	0.21	989
COL 08B	250	696	2793	0.25	2838
Mean (SEM)	118 (40)	537 (50)	2252 (250)	0.24 (0.01)	1739 (226)
p	<0.03	<0.02	NS	NS	NS
‡ Direct synthesis in LDL was calculated as the difference between the total absolute catabolic rate (observed mass x overall FCR) and the input from VLDL and IDL.					

**Table 12e.** LDL Apolipoprotein B Metabolism before and on Colestipol.(continued)

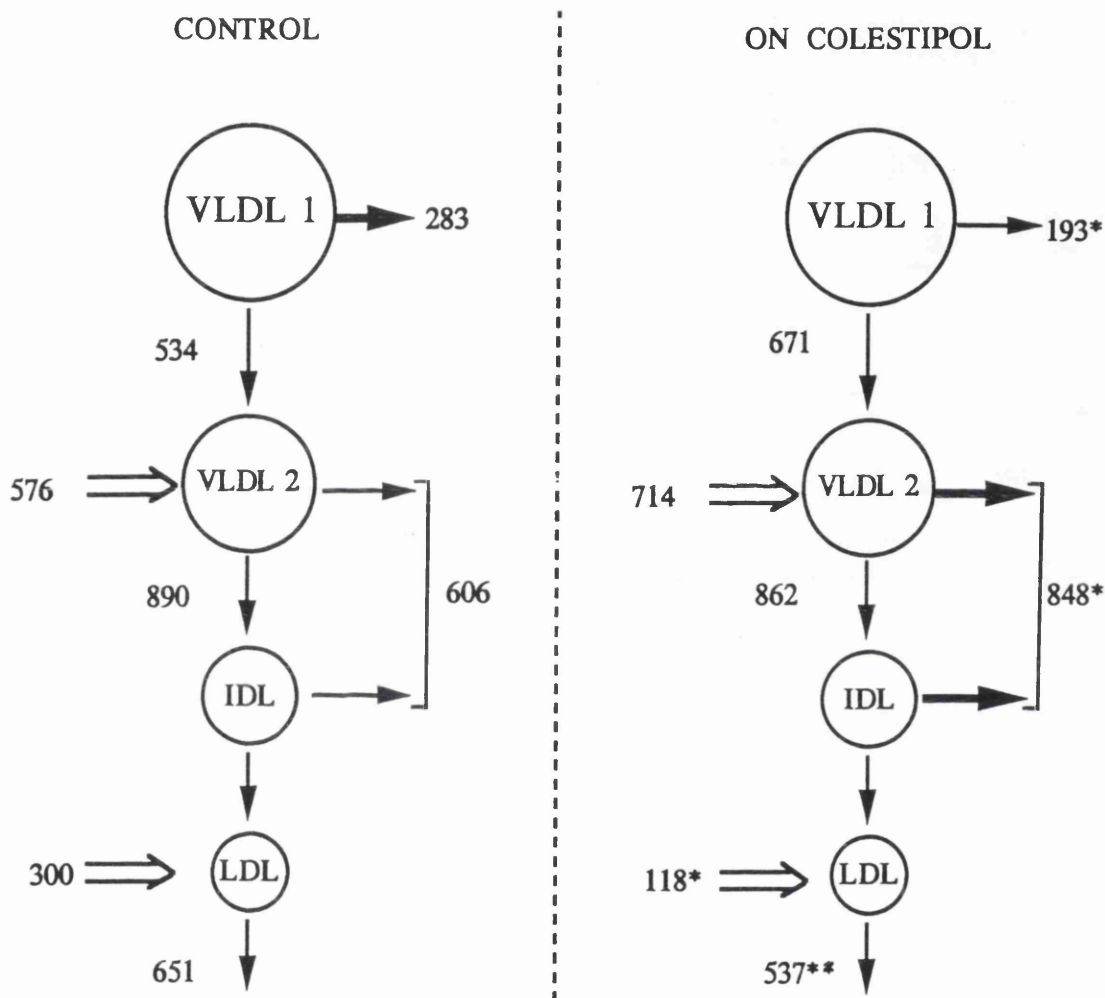
	Observed Plasma LDL pool (mg)	Total LDL Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>		
COL 01A	2629	839
COL 02A	3812	811
COL 03A	2615	759
COL 04A	3183	855
COL 05A	3226	1006
COL 06A	5096	1362
COL 07A	2342	773
COL 08A	4860	1204
Mean (SEM)	3470 (367)	951 (79)
<i>On Colestipol</i>		
COL 01B	1705	479
COL 02B	2952	854
COL 03B	1789	503
COL 04B	2837	636
COL 05B	2569	520
COL 06B	3488	806
COL 07B	2125	492
COL 08B	3792	946
Mean (SEM)	2657 (269)	655 (66)
p	p<0.002	p<0.003

significant fall in the IDL apoB plasma pool size from 781 to 678mg (13%,  $p<0.03$ ). The IDL to LDL fractional transfer rate was unaffected by drug, but the amount of LDL apoB derived from VLDL<sub>2</sub> and IDL combined fell significantly ( $p<0.02$ ). There was also a fall in the direct synthesis of LDL apoB in every subject resulting in a mean fall of 61% ( $p<0.03$ ). The calculated VLDL-derived LDL apoB plasma pool was unaffected by therapy but the observed total LDL apoB mass, which includes apoB unaccounted for by that labelled in the delipidation cascade, fell from 3427 to 2657 mg ( $p<0.002$ ). The mean fractional catabolic rate of LDL apoB, which may have been expected to rise on therapy, was unchanged as was the mean calculated total apoB synthetic rate. The observed fall in LDL, in this group of 8 subjects, may be accounted for by the reduction in total LDL apoB synthesis (flux from VLDL<sub>2</sub> and IDL plus direct synthesis) of 32% ( $p<0.003$ ), rather than any change in catabolism. Figure 37 summarises the flux of apo B through the delipidation cascade. Following therapy the direct catabolism from VLDL<sub>2</sub> and IDL density ranges was increased significantly ( $p<0.05$ ), while there was reduced clearance of VLDL<sub>1</sub> apoB and direct input of LDL apoB.

From these data it is clear that colestipol affects the kinetics of all apoB containing lipoproteins to some extent. In an attempt to correlate the changes in LDL kinetics with changes in LDL substructure, the relationship between the LDL apoB synthetic parameters and the plasma concentrations of LDL-I, II, and III was examined. In contrast to the findings reported in chapter 3 with simvastatin, no significant correlations were found between the LDL subfraction concentrations and the LDL apoB kinetic parameters, although there was a tendency for the LDL direct input to increase in accord with the levels of LDL-I and II. No such relationship could be found between LDL-III and this kinetic parameter.

#### 4.7 Discussion

The bile acid sequestrant resins are of proven efficacy in the primary and secondary prevention of CHD (Lipid Research Clinics Program 1984a, Watts *et al* 1992). In the present study, using a moderate dose of colestipol (20g.day<sup>-1</sup>), significant reductions in total cholesterol and LDL-cholesterol of 14 and 23% respectively were achieved. A non-significant increase in triglyceride was also observed consistent with other studies.



**Figure 37.** Summary of the effects of colestipol therapy on apoB metabolism. Numbers on arrows represent mass transfer of apoB in mg.d<sup>-1</sup>. \* p<0.05, \*\* p<0.02 paired student's t-test.

In order to discuss the full implications of the findings in the present study it is necessary to review more fully the known mechanisms of action of the resins. These drugs are not absorbed from the gut and act as relatively inert anion exchange resins. They exert all their effects on lipoprotein metabolism by preventing the reabsorption of bile acids, thus interrupting the enterohepatic circulation. Returning bile acids regulate the conversion of cholesterol to bile acids by negative feedback inhibition of the rate limiting enzyme, cholesterol 7 $\alpha$  hydroxylase (Reihner *et al* 1989). Interruption of the enterohepatic circulation of bile acids results in de-repression of this enzyme and a compensatory rise in both hepatic cholesterol synthesis and expression of apoB/E receptors. Kovanen *et al* (1981) first demonstrated this increased expression of receptors on hepatocytes in response to resin therapy and Reihner *et al* (1990a) confirmed these findings in humans demonstrating a more than doubling of apoB/E receptor expression in cholestyramine treated patients. At the same time Reihner and her colleagues reported concomitant 6-fold and 5-fold increases in cholesterol 7 $\alpha$  hydroxylase and HMG CoA reductase respectively in the same patients. One of the bile acids, chenodeoxycholic acid, has also been shown to be a particularly potent inhibitor of phosphatidic acid phosphatase, the pacemaker enzyme of triglyceride synthesis (Angelin, Bjorkhem & Einarsson 1986). Resin therapy, by depleting the hepatocyte of this regulatory bile acid, may therefore affect hepatic triglyceride production by releasing this enzyme from bile acid induced inhibition.

By binding irreversibly to the inert matrix of the resins, bile acids are effectively trapped in the intestinal lumen, and excreted in the faeces. Resin induced increases in faecal sterol excretion were noted by a number of early workers including, Hashim & Van Itallie (1965) and Nazir *et al* (1972). The latter group proposed as a result of their studies on plasma cholesterol kinetics that cholestyramine therapy induced an increase in endogenous synthesis of cholesterol. This was confirmed by Goodman, Noble & Dell (1973) who demonstrated an increased cholesterol production rate of 86%, ( $p < 0.01$ ) on colestipol in 7 hypercholesterolaemic subjects.

The effects of resin therapy on triglyceride turnover were examined by Angelin *et al* (1978) using tritiated glycerol. They demonstrated a significant increase in the production rate of total plasma triglyceride, but observed no change in plasma triglyceride level because of an associated increase in its fractional turnover rate. These workers concluded that those factors influencing

cholesterol and bile acid synthesis, in this case cholestyramine therapy, also affect the metabolism of triglyceride. In a refinement of this study the same workers 12 years later examined VLDL-triglyceride kinetics in a defined group of FH heterozygotes before and after cholestyramine therapy (Angelin *et al* 1990). Again they demonstrated a significant increase in VLDL-triglyceride synthesis (85%) and FCR (40%) on therapy.

The compositional changes in lipoproteins resulting from resin therapy are well documented and are thought to be partly consequent on this increased hepatic triglyceride synthesis. Witztum, Schonfeld & Weidman (1976) reported that colestipol resulted in relative triglyceride enrichment of VLDL, LDL and HDL but also noted that these changes were reversed by some compensatory mechanism as therapy was continued. In further studies (Witztum *et al* 1979) colestipol therapy was associated with reduced cholesterol:apoB ratios in all apoB containing lipoproteins. In the present study, LDL-cholesterol:apoB ratio fell by 58%. There was also a change in VLDL<sub>1</sub> and VLDL<sub>2</sub> towards larger and IDL and LDL towards smaller species. When we consider these changes alongside the significant alterations seen in the LDL subfraction profiles as a result of colestipol therapy (figure 32, table 10) it is possible that this drug is exerting two distinct effects at opposite ends on the delipidation cascade. On the one hand it is, probably by increasing triglyceride synthesis, giving rise to larger VLDL particles; and on the other it is by enhancing apoB/E receptor activity resulting in the depletion from the circulation of IDL and larger more buoyant LDL, leaving behind those smaller, denser LDL particles that correspond to LDL-III.

Building on these important findings in cholesterol and triglyceride kinetics, resin induced perturbations in the turnover of apolipoproteins were next studied. Shepherd *et al* (1979b) examined the kinetics of apoAI and apoAII in 4 hypercholesterolaemic subjects and demonstrated a significant increase in apoAI synthetic rate of 12% on cholestyramine therapy. These workers went on to examine the effects of cholestyramine on apo-LDL metabolism and showed enhanced receptor mediated clearance both in rabbits (Slater *et al* 1980) and in humans (Shepherd *et al* 1980a). The same question: what are the effects of resins on apo-LDL metabolism, was addressed using different methodology by other groups. Witztum *et al* (1985) compared apo-LDL turnover parameters in normal and cholestyramine treated guinea pigs, and reported no significant difference in LDL-FCR between the two groups. However, these workers did

demonstrate a 29% fall in apo-LDL production ( $p < 0.02$ ) on therapy. Huff *et al* (1985) examined the kinetic parameters of apoB containing lipoproteins in a group of 4 miniature pigs. Again, this group found no significant change in LDL-FCR, but reported a marked fall in LDL direct synthesis by 51% ( $p < 0.005$ ), and no change in any VLDL kinetic parameters. These studies, including the present one, may seem to offer conflicting answers to the question of the effect of resin therapy on LDL-FCR. However, these findings may not necessarily be at variance when we consider the following arguments.

After studying the effect of resin therapy on LDL composition and metabolism in humans, Young *et al* (1989) argued that drug induced enhancement of apoB/E receptor activity is incompatible with a reduction or a static response in LDL-FCR. There are a number of possible solutions to this apparent paradox. Firstly, the apoB/E receptors are responsible not only for the catabolism of LDL particles, but potentially of all apoB containing lipoproteins. In the present study and that presented in chapter 3, drugs known to enhance receptor activity are shown to increase VLDL<sub>2</sub> and IDL apoB clearance. Such receptor mediated removal of LDL precursors is certainly one possible explanation of resin induced reduction of plasma LDL-cholesterol levels with no apparent rise in LDL apoB FCR. In the patient group studied here, no overall increase in LDL-FCR was demonstrated. Shepherd *et al* (1980a) reported that the cholestyramine induced reduction in LDL-cholesterol was solely attributable to enhanced receptor mediated clearance of apo-LDL. However, other studies described above have failed to demonstrate an increase in LDL-FCR attributable to resin therapy (Witztum *et al* 1985, Huff *et al* 1985). This variability in response to resin therapy may be explained when the subjects studied and the methodology used are considered. Shepherd *et al* (1980a) studied a group of 5 women heterozygous for FH; a group therefore, with a specific genetic defect of apoB catabolism. Such patients respond to resin therapy in the same way as they respond to statin therapy (Bilheimer *et al* 1983) by predominantly increasing their LDL clearance. A more heterogeneous group of hypercholesterolaemic patients such as that studied here shows a variety of kinetic responses perhaps determined by the nature of their underlying metabolic defect. Witztum *et al* (1985) and Huff *et al* (1985) studied normolipidaemic animals, presumably without any underlying catabolic defect. Witztum *et al* (1985) and Young *et al* (1989) have used an alternative to traditional methodology by altering their study protocols to take account of drug induced perturbations of the tracer. By isolating LDL in a control phase and in a drug treated phase and reinjecting

these labelled autologous lipoproteins simultaneously in a second drug treated phase they demonstrated, both in animals and in humans, the metabolic importance of drug-induced changes in LDL composition. Young *et al* (1989) suggest that the lack of observed change in LDL-FCR on resin therapy is a function of the altered tracers used in most kinetic experimental protocols. The FCR of smaller, denser LDL, which is the product of resin therapy, is reduced if anything when compared to the FCR of larger more buoyant LDL, which would have been isolated and used as tracer in the control phase of most LDL turnovers. The compositional changes in LDL and the associated changes in LDL subfraction profile observed in the present study would confirm these findings. However, by injecting a labelled precursor of LDL, as has been done in the VLDL turnover protocol described and used here, such obvious effects are largely, though not completely, avoided.

In the present study, in accord with many other workers the observed LDL apoB mass could not be accounted for by that entering the LDL density interval from the delipidation cascade alone. The source of this additional mass can only be guessed at using the present methodology and is usually ascribed to *de novo* or direct LDL synthesis. Whatever its source this LDL is unlabelled and therefore its FCR cannot be computed. There is a possibility that this portion of LDL may have a greatly enhanced FCR as a result of resin therapy or that its synthesis rate is reduced. Although the latter is suggested in the kinetic analysis above the former cannot be excluded.

One other explanation of the reduction in LDL-cholesterol with resin therapy also centres on its precursors. During resin therapy there is increased hepatic synthesis and secretion of triglyceride. The larger triglyceride-enriched VLDL particles that the liver secretes into the circulation have been shown by others (Packard *et al* 1984) to be removed prematurely from the circulation before reaching the LDL density interval. Such a shift in triglyceride metabolism may explain the compositional changes observed in this study and in others, and may be at least a contributory factor in the observed fall in plasma LDL level.

In conclusion, colestipol therapy in moderate hypercholesterolaemia is associated with changes throughout the apoB containing lipoproteins in terms of their composition and their kinetic parameters. In the latter, there is some degree of similarity with simvastatin (chapter 3). If we assume that both drugs, albeit by fundamentally different means, enhance apoB/E receptor activity these kinetic similarities may be readily explained.



## Chapter 5 *Acipimox*

I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Isaac Newton

### 5.1. Introduction

Acipimox is a structural analogue of nicotinic acid (figure 9) first described in 1980 (Ambrogi *et al* 1980). It was introduced into clinical practice in an attempt to reproduce the effective lipid lowering qualities of its parent compound but without the significant adverse side effect profile associated with nicotinic acid.

A comparison of the antilipolytic activity of nicotinic acid and acipimox in humans by Fuccella *et al* (1980) has shown acipimox to be approximately 20 times as potent as its parent compound. This means that acipimox has been an effective lipid lowering agent at milligram doses, rather than the gram doses of nicotinic acid required for pharmacological action.

Inhibition of the hormone sensitive triglyceride lipase present in adipose tissue is thought to be the primary action of acipimox and this is described above in section 1.6.2. Other actions have also been proposed for acipimox and these are pertinent

to the present study. The effects of acipimox on HDL-cholesterol levels and more specifically the alteration in the HDL<sub>2/3</sub> ratio prompted a number of workers to suggest that this drug might affect the balance between lipoprotein lipase and hepatic lipase activities.

Sirtori *et al* (1981) initially suggested that acipimox may act as a promotor of lipoprotein lipase activity. However, two groups (Stuyt *et al* 1985, Taskinen & Nikkila 1988), studying different types of hyperlipidaemic patients, did not support this. Stuyt *et al* (1985) showed no change in lipoprotein lipase activity associated with acipimox therapy in type III and type IV hyperlipoproteinaemic patients, but did report a significant decrease in hepatic lipase activity with this drug. Similarly, Taskinen & Nikkila (1988) could demonstrate no significant change in post heparin plasma lipoprotein lipase activity on acipimox therapy in type IV and type V patients, but did show a reduction of 25% in hepatic lipase activity. Although no direct, kinetic data on the actions of acipimox was available to the latter workers they went on to hypothesise that a reduction in VLDL production was probably the main mechanism of LDL lowering in their patients treated with acipimox.

The aim of the present study was to examine in detail the mechanism of action of the nicotinic acid analogue, acipimox, in patients with primary, moderate hypercholesterolaemia and in particular to investigate the mechanisms behind the LDL-cholesterol lowering capacity of this drug. The same dual tracer VLDL<sub>1</sub> and VLDL<sub>2</sub> turnover technique as used in chapters 3 and 4 was employed, as were the same ultracentrifugal analyses to examine the effects of acipimox therapy on the lipoprotein subfraction profiles. Drug induced changes in lipoprotein composition and in LDL subfraction distribution were noted and, again, as in chapter 4 multiple effects were observed on the delipidation chain from VLDL<sub>1</sub> to LDL. The known effects of this drug on hepatic triglyceride synthesis and its inhibition of the enzyme hepatic lipase are invoked to explain those changes observed in the apoB kinetic parameters.

## 5.2. Protocol

This study of the effects of acipimox on the metabolism of apoB containing particles consisted of three phases :

1. Preliminary Screening Period. During this 5 week run in period each patient was screened for cardiological, haematological, hepatic, endocrine, renal, and metabolic disease by routine clinical and laboratory testing.
2. Baseline Study. In this period a VLDL turnover study was performed on each patient to serve as a control. Baseline lipid, lipoprotein and lipoprotein subfractions were measured as described above.
3. Active Treatment Period. Immediately after the baseline assessment of apolipoprotein B metabolism the patients were commenced on acipimox therapy rising to a dose of 1250mg. d<sup>-1</sup> over a period of 7-10 days. Patients remained on this dose for 10 weeks. During the final 2 weeks each patient underwent a second turnover investigation while on acipimox therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated.

### 5.3 Subjects

Seven study patients were selected from the Risk Factor Clinic at Glasgow Royal Infirmary. All patients had an initial elevated total cholesterol level ( $\geq 7.0\text{mmol.L}^{-1}$ ) despite adherence to a standard lipid lowering diet (Study Group, European Atherosclerosis Society dietary recommendations, 1988) and a triglyceride level less than  $3.0\text{mmol.L}^{-1}$ , but did not have clinical evidence of FH. Patients were screened for cardiological, haematological, hepatic, endocrine, renal and metabolic disease by routine clinical and laboratory testing to exclude secondary causes of hyperlipidaemia and any illness thought to influence the outcome of the study. Pre-menopausal women were excluded from the study. The characteristics of each patient are summarised in table 13. Three of the subjects studied were receiving prescribed medications for concurrent medical problems. All prescribed medications were continued unchanged throughout the course of the study.

### 5.4 Adverse Events

Acipimox was reasonably well tolerated by all subjects participating in the study. All patients reported initial facial and neck flushing after the first few doses. This resolved after the first 3 days of therapy in all but one patient. In patient APX 01, this drug induced vasodilatation continued throughout the course of the study but did not force the patient to discontinue or interrupt his therapy. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy.

Table 13. Summary of Patient Characteristics

Subject	Sex‡	Age§	BMI	Apo E Phenotype	Concomitant Drug Therapy	Clinical Diagnoses¶
APX 01	M	36	29.8	3/3	Nil	Nil
APX 02	M	59	24.8	3/3	Nil	Nil
APX 03	F	41	23.3	3/3	Bendofluazide, Enalapril maleate	Hypertension
APX 04	F	47	23.5	3/3	Nil	Post menopausal bleeding
APX 05	M	49	27.7	3/3	Glyceryl trinitrate	Angina
APX 06	F	52	20.8	3/3	Nifedipine	Angina
APX 07	F	60	26.0	3/3	Nil	Migraine

‡Male (M), Female (F). §Ages in years at start of study. || Body mass index (BMI) calculated from weight (kg)/height<sup>2</sup>(m). ¶Current medical problems of subjects at start of study.

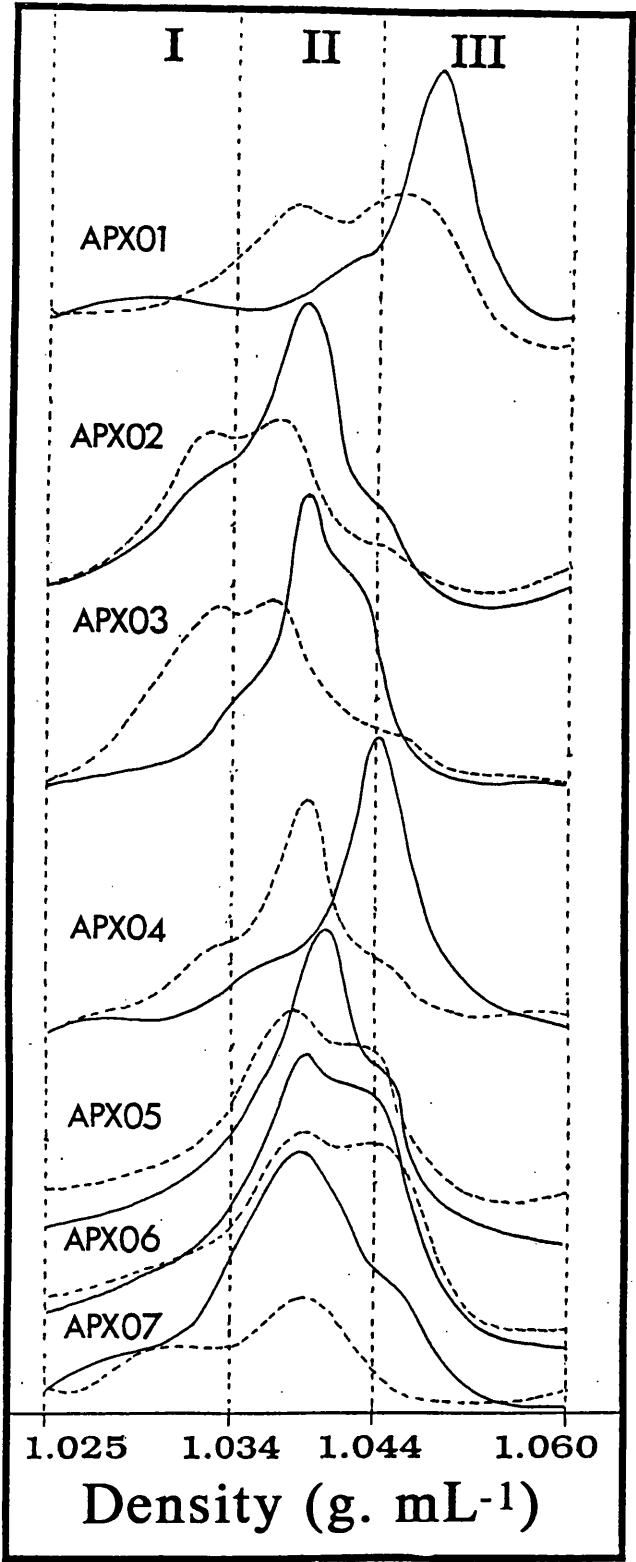
## 5.5 Lipids and Lipoproteins

Plasma lipid and lipoprotein levels of those patients studied are shown in table 14. Treatment reduced plasma cholesterol by 18% ( $p < 0.001$ ). This decrement was due, in this group, to a 20% fall in LDL-cholesterol ( $p < 0.002$ ), while VLDL and HDL-cholesterol were unchanged. There was also a non-significant fall in triglyceride of 26%. Acipimox therapy had no significant effect on plasma Lp(a). Centrifugal analysis of the subfraction distribution in the HDL and LDL density intervals was performed. The HDL<sub>2/3</sub> ratio increased by 35% due to a 27% rise in HDL<sub>2</sub>, but the scatter of responses did not allow this to achieve statistical significance. Figure 38 shows the LDL subfraction profiles obtained upon density gradient fractionation of subjects' plasma. As before (chapters 3 and 4), there was some inter-individual variation in the pattern observed, but in most three distinct populations of particles could be distinguished both before and during therapy. The increment in the least dense LDL-I species was the most significant change in these subjects (57%,  $p < 0.05$ ). LDL-II was unaffected by therapy but LDL-III fell by 50%. These acipimox induced responses resulted in a general shift from LDL-III to LDL-I, i.e. from small, dense LDL to larger, more buoyant species in LDL-III (figure 38, table 15). Compositional analysis of the four major apoB containing lipoprotein classes (table 16) demonstrated that acipimox had significantly altered the make-up of these species. There appeared to be a general fall in the lipid components of all apoB containing particles with the most significant changes occurring in VLDL<sub>2</sub>, where there were significant falls in the absolute content of free and esterified cholesterol ( $p < 0.01$ ,  $p < 0.005$ ), triglyceride ( $p < 0.005$ ), phospholipid ( $p < 0.05$ ) and protein ( $p < 0.05$ ). The core content of IDL and LDL became relatively cholesteryl ester enriched, with the cholesteryl ester/triglyceride ratio increasing from 3.4 to 3.9 in IDL and 7.2 to 8.3 in LDL.

## 5.6 Apolipoprotein B Metabolism

The influence of acipimox therapy on apolipoprotein B metabolism was examined before and on drug (representative decay curves for one subject, APX 03 are shown in figures 39-42). Before therapy radioactive apoB disappeared rapidly from the VLDL<sub>1</sub> flotation interval and appeared in VLDL<sub>2</sub> over a period of 4-6h after injection (figure 39). The decay of radioactive apoB in VLDL<sub>2</sub> was much slower than in VLDL<sub>1</sub>, taking approximately 60 hours to reduce to 1% of the injected dose (figure 40). Acipimox therapy appeared to reduce the clearance rate of VLDL<sub>1</sub> apoB (figures 41) but increase that of VLDL<sub>2</sub> apoB (figures 42). The flux





**Figure 38.** LDL subfraction profiles at baseline (—) and during acipimox therapy (---) in subjects APX 01-APX 07.

**Table 15.** LDL Subfraction Changes with Acipimox Therapy

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100mL plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
APX 01A	468	14	73	381
APX 02A	337	84	221	32
APX 03A	597	99	385	113
APX 04A	430	0	79	351
APX 05A	280	32	216	32
APX 06A	460	64	299	97
APX 07A	340	63	225	52
Mean (SEM)	416 (40.3)	51 (13.8)	214 (42.2)	151 (56.8)
<i>On Acipimox</i>				
APX 01B	466	18	248	200
APX 02B	360	149	171	40
APX 03B	378	154	180	44
APX 04B	474	107	311	56
APX 05B	376	54	233	89
APX 06B	327	42	194	91
APX 07B	309	133	164	12
Mean (SEM)	384 (24.1)	94 (20.9)	214 (20.0)	76 (23.2)
p*	NS	<0.05	NS	NS

\* Comparison of the means was performed using paired students t-test.

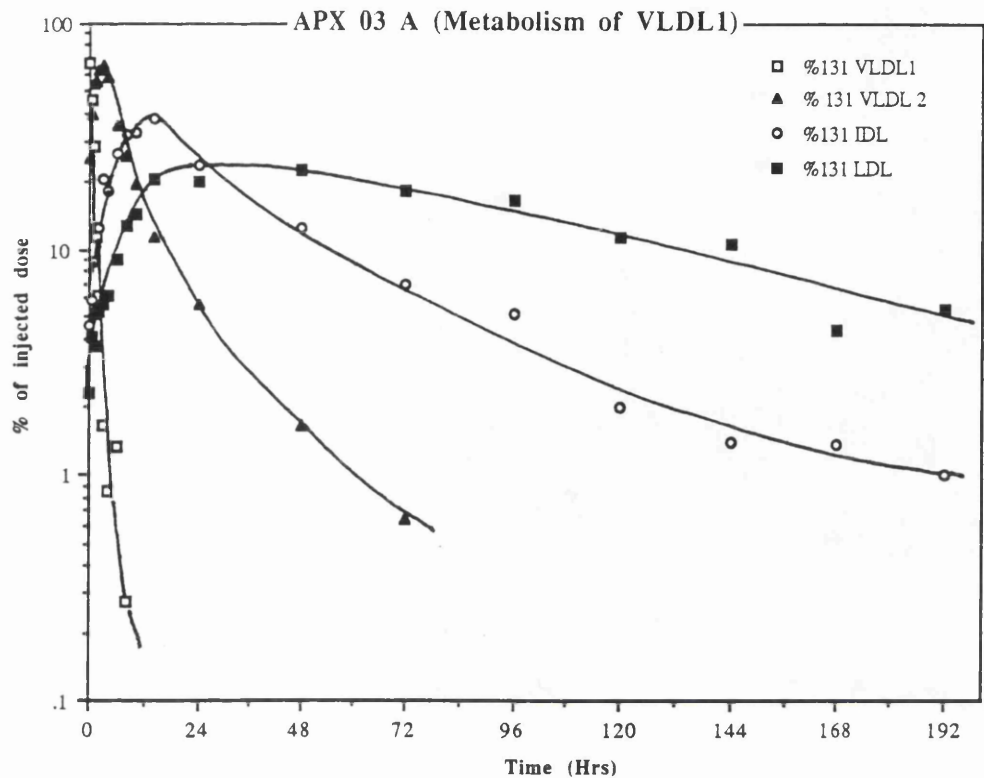
of radioactive apoB through the IDL density interval was very different when the effects of acipimox on IDL derived from VLDL<sub>1</sub> and that derived from VLDL<sub>2</sub> were compared. Therapy did not significantly alter the peak height or shape of the IDL apoB decay curve derived from VLDL<sub>2</sub>, but caused a drop in the maximum reached by VLDL<sub>1</sub>-derived IDL apoB from 40 to 25% of injected dose and markedly delayed the flux of this portion of radioactive IDL apoB into LDL. Before therapy, LDL apoB radioactivity reached a maximum of 40% of injected dose about 24 h after VLDL<sub>2</sub> injection (figure 40), whereas during therapy (figure 42)



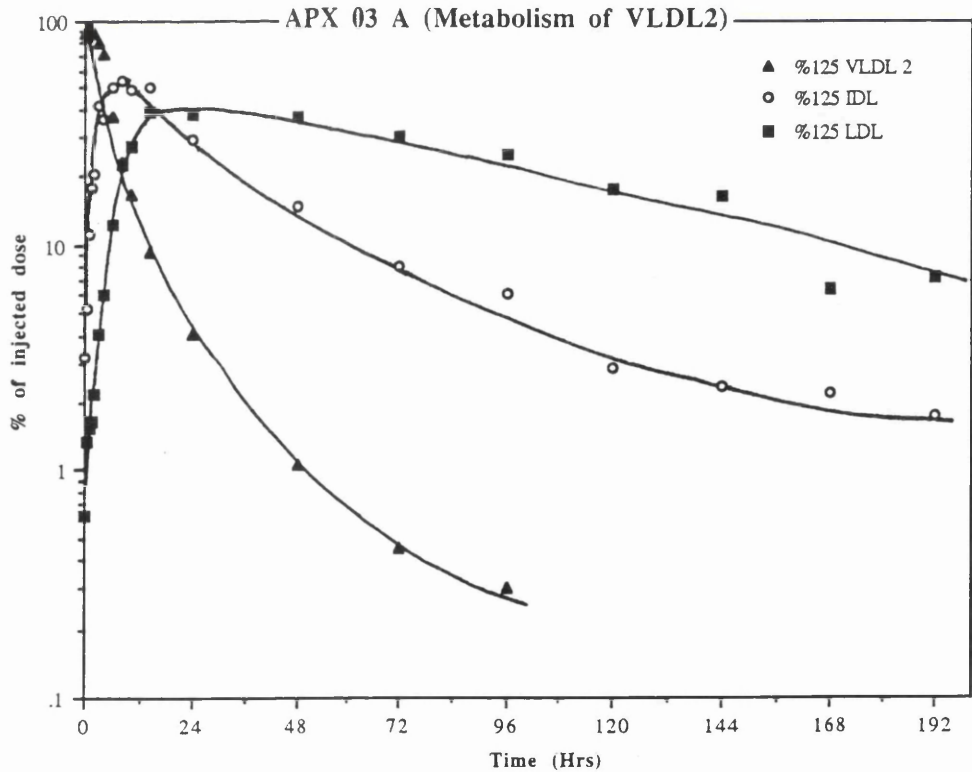
**Table 16. Composition of Apolipoprotein B Containing Lipoproteins Before and On Acipimox Therapy.**

	Free Cholesterol	Cholesteryl Ester	Triglyceride (mg. dL <sup>-1</sup> )	Phospholipids	ApoB
VLDL <sub>1</sub>	Before Therapy On Therapy	5.33 (2.09)* 4.19 (0.86)	81.20 (15.86) 65.43 (12.88)	23.79 (4.69) 17.29 (3.34)§	4.95 (0.74) 3.51 (0.83)§
VLDL <sub>2</sub>	Before Therapy On Therapy	6.05 (0.66) 3.81 (0.52)‡	32.05 (2.46) 20.79 (2.33)†	22.36 (3.47) 13.11 (1.32)§	7.40 (0.45) 4.46 (0.66)§
IDL	Before Therapy On Therapy	8.09 (0.81) 7.41 (1.59)	11.80 (1.06) 8.97 (0.62)§	25.39 (4.54) 19.50 (1.13)	10.43 (0.92) 13.84 (2.72)
LDL	Before Therapy On Therapy	43.10 (5.07) 34.05 (5.14)	21.62 (1.80) 15.30 (0.76)†	86.21 (6.82) 69.36 (4.77)§	104.34 (11.03) 73.93 (3.74)

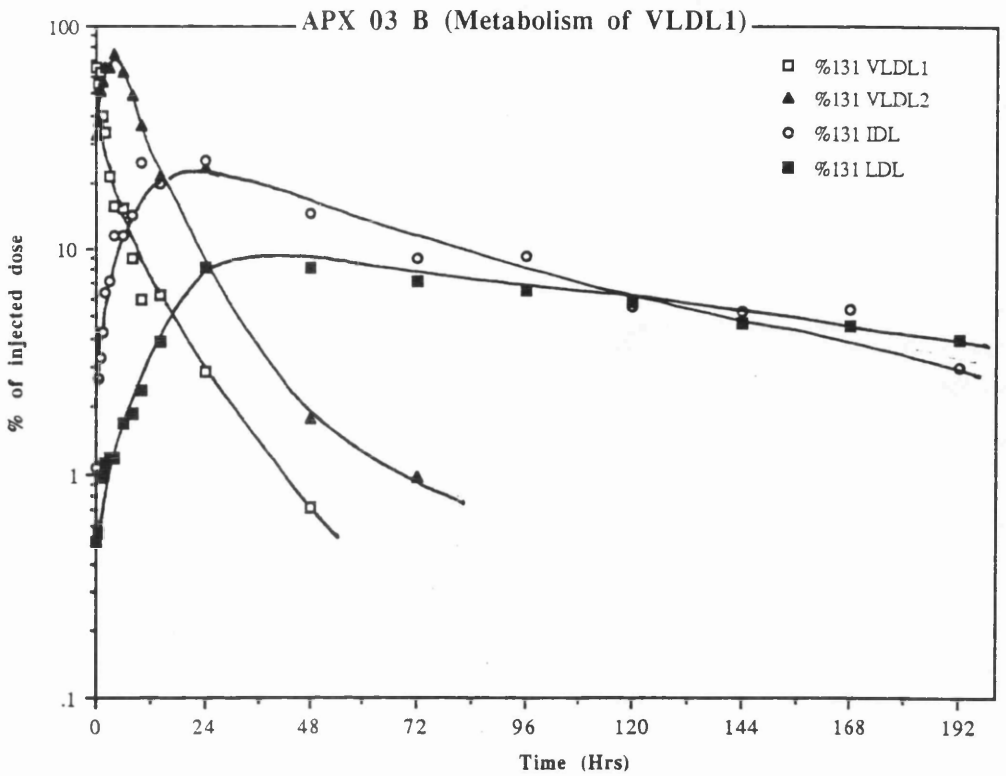
\* Mean (SEM)  
† Significantly different from before therapy, †p<0.005, ‡p<0.01, §p<0.05 by students paired t-test



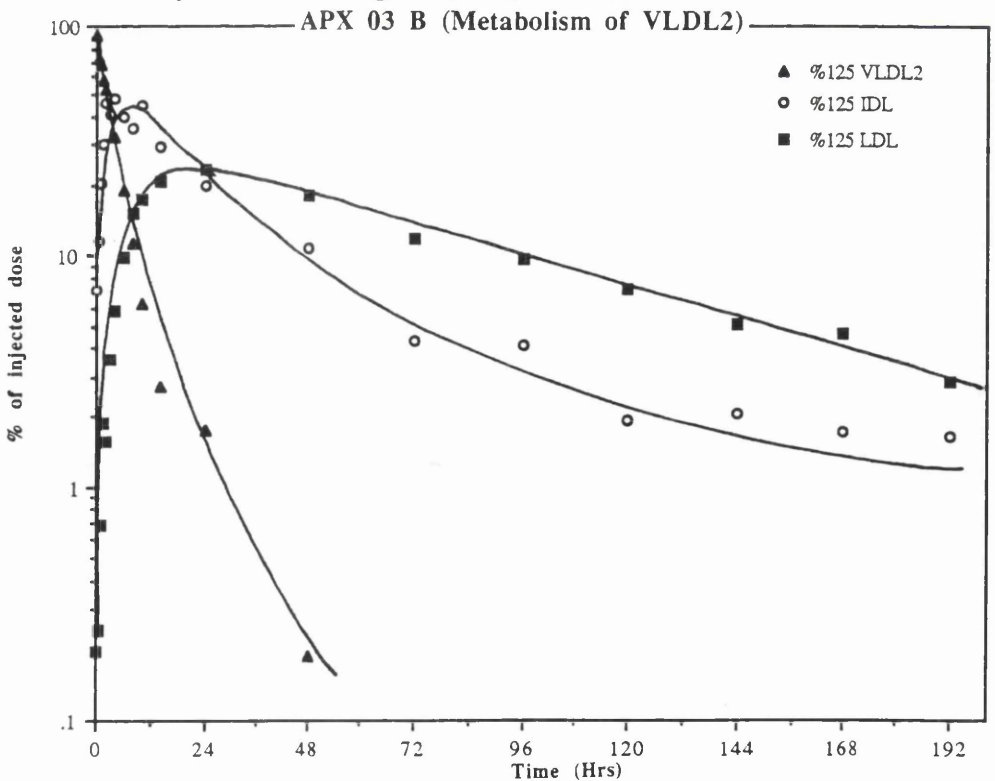
**Figure 39.** Baseline. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure 40.** Baseline Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure 41.** On Acipimox. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>



**Figure 42.** On Acipimox. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

**Table 17a** VLDL<sub>1</sub> Apolipoprotein B Metabolism before and on Acipimox

Subject	Synthesis (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To VLDL <sub>2</sub> (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
APX 01A	857	239	0.62	2.97
APX 02A	1504	122	7.83	4.49
APX 03A	870	49	9.29	8.46
APX 04A	452	126	1.76	1.82
APX 05A	905	264	1.59	1.84
APX 06A	392	93	1.33	2.89
APX 07A	352	77	1.47	3.11
Mean (SEM)	762 (154)	139 (31)	3.41 (1.35)	3.65 (0.87)
<i>On Acipimox</i>				
APX 01B	860	148	1.86	3.95
APX 02B	220	62	1.42	2.13
APX 03B	136	20	2.23	4.59
APX 04B	951	122	4.00	3.80
APX 05B	263	58	0.01	4.53
APX 06B	364	87	2.10	2.08
APX 07B	1100	58	13.59	5.38
Mean (SEM)	556 (151)	79 (16)	3.60 (1.72)	3.78 (0.47)
p *	NS	NS	NS	NS
* Differences between the time points were assessed by the paired t-test.				

**Table 17b.** VLDL<sub>2</sub> Apolipoprotein B Metabolism before and on Acipimox

	Direct Synthesis (mg.d <sup>-1</sup> )	Flux from VLDL <sub>1</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux to IDL+ LDL (pools.d <sup>-1</sup> )
<i>Before Therapy</i>					
APX 01A	626	710	684	0.56	1.30
APX 02A	928	548	486	1.51	1.52
APX 03A	492	415	244	0.44	3.24
APX 04A	640	230	322	0.13	2.57
APX 05A	1178	484	559	0.59	2.39
APX 06A	279	269	251	0.32	1.86
APX 07A	728	239	355	0.81	1.93
Mean (SEM)	696 (110)	414 (68)	414 (63)	0.62 (0.17)	2.12 (0.25)
<i>On Acipimox</i>					
APX 01B	784	585	369	0.76	2.95
APX 02B	1231	132	257	1.94	3.37
APX 03B	756	92	112	0.51	7.13
APX 04B	830	463	296	0.02	4.35
APX 05B	932	263	340	0.08	3.45
APX 06B	481	181	257	0.58	2.00
APX 07B	901	312	184	2.93	3.65
Mean (SEM)	845 (85)	290 68	259 (34)	0.97 (0.41)	3.84 (0.61)
p	NS	NS	<0.02	NS	<0.01

**Table 17c.** IDL Apolipoprotein B Metabolism before and on Acipimox.

	Flux from VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
APX 01A	887	978	0.32	0.59
APX 02A	734	1023	0.22	0.50
APX 03A	769	679	0.29	0.84
APX 04A	825	716	0.40	0.76
APX 05A	1332	970	0.25	1.12
APX 06A	462	519	0.30	0.59
APX 07A	682	677	0.18	0.83
Mean (SEM)	813 (100)	795 (73)	0.28 (0.03)	0.75 (0.08)
<i>On Acipimox</i>				
APX 01B	1084	861	0.39	0.87
APX 02B	856	1053	0.30	0.51
APX 03B	795	454	0.74	1.02
APX 04B	1288	1273	0.67	0.34
APX 05B	1171	711	0.73	0.91
APX 06B	513	526	0.11	0.86
APX 07B	648	898	0.30	0.42
Mean (SEM)	908 (107)	825 (109)	0.46 (0.09)	0.70 (0.10)
p	NS	NS	NS	NS

**Table 17d.** LDL Apolipoprotein B Metabolism before and on Acipimox.

	Direct Synthesis ‡ (mg.d <sup>-1</sup> )	Flux from IDL + VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	VLDL derived Plasma Pool (mg)	Fractional Catabolic Rate (pools.d <sup>-1</sup> )	Total ApoB Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>					
APX 01A	297	577	2469	0.23	1780
APX 02A	794	514	1378	0.37	3226
APX 03A	326	592	2921	0.20	1688
APX 04A	53	542	2062	0.26	1145
APX 05A	476	1090	2935	0.37	2559
APX 06A	145	311	1422	0.22	816
APX 07A	153	563	3139	0.18	1233
Mean (SEM)	321 (95)	598 (89)	2332 (276)	0.26 (0.03)	1778 (321)
<i>On Acipimox</i>					
APX 01B	9	752	2892	0.26	1653
APX 02B	162	548	1703	0.32	1613
APX 03B	132	464	1362	0.34	1024
APX 04B	92	437	2064	0.21	1873
APX 05B	327	651	2138	0.30	1522
APX 06B	251	456	1327	0.34	1096
APX 07B	93	402	2616	0.15	2094
Mean (SEM)	152 (40)	530 (49)	2015 (226)	0.27 (0.03)	1554 (146)
p	NS	NS	NS	NS	NS
‡ Direct synthesis in LDL was calculated as the difference between the total absolute catabolic rate (observed mass x overall FCR) and the input from VLDL and IDL.					

**Table 17e.** LDL Apolipoprotein B Metabolism before and on Acipimox (continued)

	Total Plasma LDL pool (mg)	Total LDL Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>		
APX 01A	3759	874
APX 02A	3524	1308
APX 03A	4527	918
APX 04A	2263	595
APX 05A	4216	1566
APX 06A	2082	456
APX 07A	3991	716
Mean (SEM)	3480 (359)	919 (149)
<i>On Acipimox</i>		
APX 01B	2926	761
APX 02B	2207	710
APX 03B	1750	596
APX 04B	2500	529
APX 05B	3227	978
APX 06B	2065	707
APX 07B	3238	495
Mean (SEM)	2559 (222)	682 (62)
p	<0.05	NS



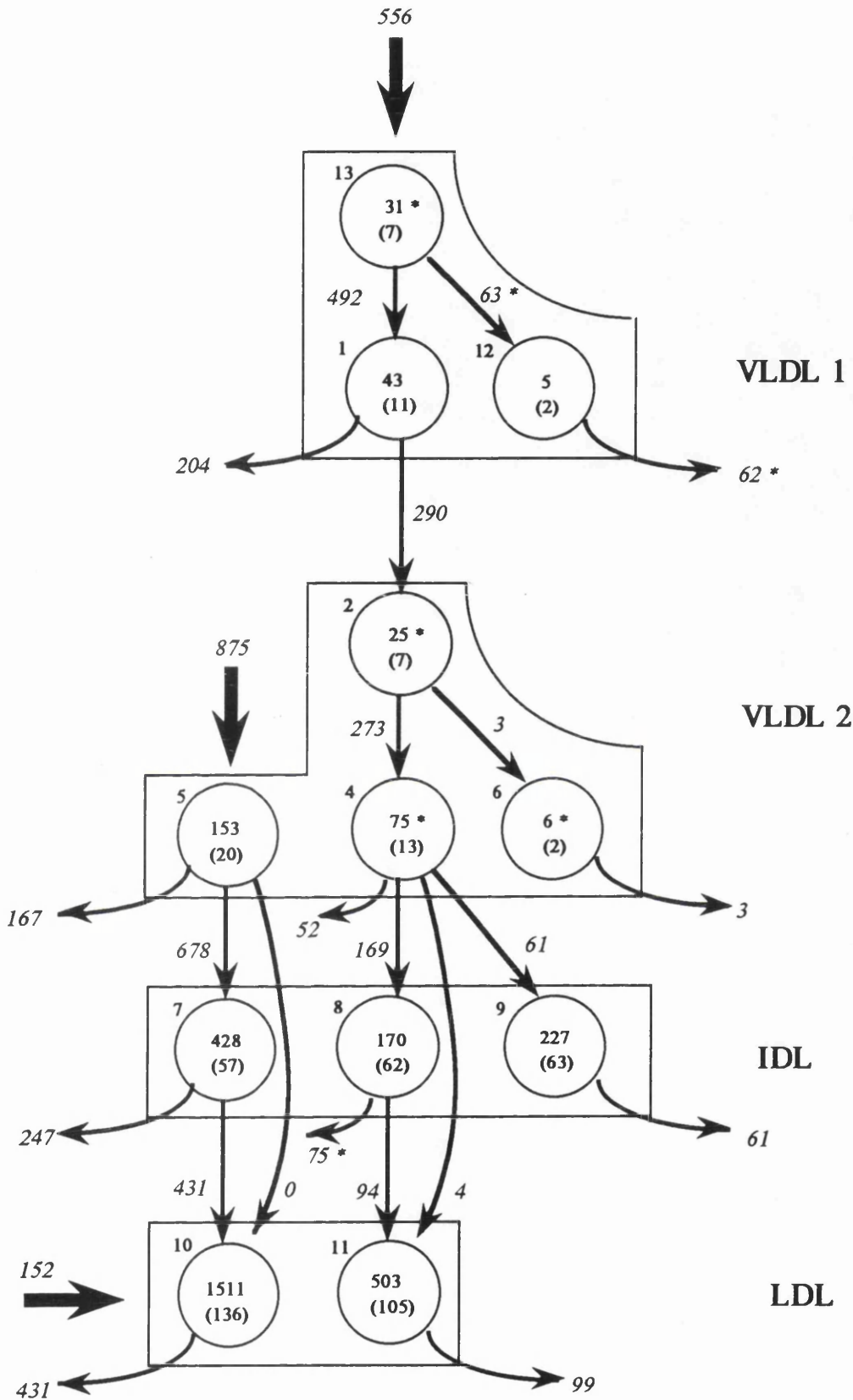
the LDL apoB maximum was only 25%. In this subject (APX 03) the decay rates of LDL apoB before and during therapy were virtually super-imposable suggesting that the clearance rate of LDL apoB was unchanged by acipimox therapy.

Kinetic rate constants and apoB fluxes were derived by compartmental modelling as described in chapter 2. The results for all 7 subjects are summarised in table 17; decay curves for the other 6 subjects and individual kinetic constants and masses for all patients are shown in appendix 4.

VLDL<sub>1</sub> apoB pool size fell by 43%, from 762 to 556mg, and this was associated with a 27% fall in apoB synthesis, while the mean direct fractional catabolic rate of VLDL<sub>1</sub> apoB and fractional transfer rate to VLDL<sub>2</sub> were unchanged by therapy. The VLDL<sub>2</sub> apoB pool size was diminished on drug therapy by 37% ( $p<0.02$ ). Increases in VLDL<sub>2</sub> apoB direct input in 6 out of 7 subjects led to a mean increment in this parameter by 21%. VLDL<sub>2</sub> apoB fractional transfer to IDL and LDL was significantly increased (81%,  $p<0.01$ ) by acipimox therapy but there was no significant increase in IDL apoB pool size due to an associated increase in the direct catabolic rate of this species (65%). The IDL to LDL fractional transfer rate was unaffected by drug, as was the amount of LDL apoB derived from VLDL<sub>2</sub> and IDL combined. There was also a non-significant fall in the direct synthesis of LDL apoB (52%). The calculated VLDL-derived LDL apoB plasma pool was unaffected by therapy but the total LDL apoB mass, which includes apoB unaccounted for by that labelled in the delipidation cascade, fell from 3480 to 2559mg ( $p<0.05$ ). The mean fractional catabolic rate of LDL apoB was unchanged as was the mean calculated total apoB synthetic rate. The observed fall in LDL, in this group of 7 subjects, may be accounted for by the reduction in total LDL apoB synthesis (flux from VLDL<sub>2</sub> and IDL plus direct synthesis) of 26% ( $p=0.08$ ), rather than any change in catabolism. Figures 43 and 44 summarise the flux of apo B through the delipidation cascade. In contrast to the findings with simvastatin and colestipol reported in chapters 3 and 4 respectively, there was no significant increase in the combined, direct catabolism from VLDL<sub>2</sub> and IDL density ranges following acipimox therapy.

From these data it is clear that acipimox significantly affects the kinetics of both VLDL apoB fractions studied and, in turn, the synthesis of LDL apoB. In an attempt to correlate the changes in LDL kinetics with changes in LDL substructure, the relationship between the LDL apoB synthetic parameters and the plasma concentrations of LDL-I, II, and III was examined. Again, in contrast to the





**Figure 44.** Summary of the effects of acipimox therapy on apoB metabolism. Numbers in circles represent apoB pool size [mean (SEM)] in mg. Numbers on arrows represent mean transfer of apoB in mg.d<sup>-1</sup>. \* significantly different from control p<0.05.

findings reported in chapter 3 with simvastatin, no significant correlations were found between the LDL subfraction concentrations and the LDL apoB kinetic parameters in these patients.

## 5.7 Discussion

Acipimox, at a dose of 1250mg. d<sup>-1</sup> in this group of moderately hypercholesterolaemic subjects, produced significant reductions of total cholesterol and LDL-cholesterol of 18 and 20%, respectively. These lipid and lipoprotein changes are consistent with the findings of Crepaldi *et al* (1988) who noted falls of 16 and 18% in the same lipid parameters using the slightly reduced dose of 1200mg. d<sup>-1</sup> in type II hyperlipoproteinaemic patients. These workers also demonstrated significant falls in plasma triglyceride level with acipimox in type IV, but not type II patients. Similarly, HDL-cholesterol was only noted to rise as a result of therapy in hypertriglyceridaemic subjects, but, interestingly, Taskinen & Nikkila (1988) failed to demonstrate any significant short-term effect on HDL-cholesterol or the subfractions HDL<sub>2</sub> and HDL<sub>3</sub>, even in severely hypertriglyceridaemic patients. Ball *et al* (1986) reported similar findings with a dose of 750mg. d<sup>-1</sup> and concluded that the fall in plasma triglyceride was most marked in those patients with an initial triglyceride level in excess of 3mmol. L<sup>-1</sup>. All patients in the present study fell below this cut-off and although a fall in triglyceride was observed in the group as a whole this was not statistically significant.

Acipimox has been shown to be a potent inhibitor of the hormone-sensitive lipase in adipose tissue (Stirling *et al* 1985). Because the breakdown of stored triglyceride is reduced there is also a reduction in the release of non-esterified fatty acids into the circulation (Fuccella *et al* 1980, Vaag *et al* 1991) and consequently reduced availability of substrates for hepatic triglyceride synthesis (Carlson & Oro 1962; Stirling *et al* 1985).

Because of this reduction in triglyceride synthesis there appears, as observed here, to be a shift in the pattern of hepatic VLDL production from large, triglyceride-rich particles (VLDL<sub>1</sub>) to smaller, relatively triglyceride-poor species (VLDL<sub>2</sub>). This results in a reduction in the contribution that VLDL<sub>1</sub> apoB makes to VLDL<sub>2</sub> apoB with acipimox therapy. From the present study, VLDL<sub>2</sub> apoB is transferred at an increased rate into the IDL density interval. This may be explained by either, or both, of the following reasons. Firstly, VLDL<sub>1</sub> being relatively triglyceride poor will be more efficiently delipidated to form IDL, even in the face of unaltered

lipoprotein lipase activity (Taskinen & Nikkila 1988, Stuyt *et al* 1985). Alternatively, if the VLDL<sub>2</sub> fractional transfer rate to IDL is regarded as a composite of a slow component (VLDL<sub>1</sub>-derived VLDL<sub>2</sub> going to IDL) and a fast component (directly synthesised VLDL<sub>2</sub> going to IDL), any alteration in VLDL<sub>1</sub> apoB synthesis will shift the composite FTR towards the faster pathway by reducing VLDL<sub>1</sub> to VLDL<sub>2</sub> transfer (figures 43 and 44).

When the VLDL<sub>2</sub> to IDL to LDL pathway (i.e. the delipidation chain derived from directly synthesised VLDL<sub>2</sub>) is examined (figure 43 and 44) it is clear that this is relatively unaffected by acipimox therapy while those masses of VLDL<sub>2</sub>, derived from VLDL<sub>1</sub>, and their products in the IDL and LDL density intervals, are markedly diminished.

Such a drug-induced shift in VLDL synthesis, resulting in a larger proportion of circulating VLDL<sub>2</sub> would perhaps be expected to result in an expanded LDL apoB pool, for Packard *et al* (1984) have demonstrated that small VLDL<sub>2</sub> rather than VLDL<sub>1</sub> is the main precursor of plasma LDL derived from delipidation. This indeed would probably be the case with acipimox therapy if it were not for this drug's other effects. Acipimox has been shown to inhibit hepatic triglyceride lipase (Taskinen & Nikkila 1988, Stuyt *et al* 1985). Studies of apoB metabolism in subjects with hepatic lipase deficiency (Demant *et al* 1988) have demonstrated that this enzyme is responsible for the delipidation of IDL, and therefore the conversion of IDL to LDL. The action of acipimox, in inhibiting hepatic lipase, is to cause a hold-up in the transfer of apoB down the delipidation cascade from IDL to LDL. This results in reduced synthesis of LDL and is one of the identified causes for reduced LDL levels in the subjects studied here. In addition, the reduced VLDL<sub>1</sub> apoB synthesis, described above results in decreased input of material into the chain reducing the masses of all compartments derived from VLDL<sub>1</sub> (figure 44). Finally, there appears to be a reduction in the circulating LDL mass because of drug-induced suppression of direct LDL synthesis.

To explain the mechanism by which acipimox lowers the plasma LDL-cholesterol level in hypercholesterolaemic subjects was one of the main objectives of the present study. There is a very limited literature in this area and many of the suggested mechanisms of action proposed for acipimox are based on indirect evidence or extension of work done in the past on nicotinic acid.

Arguing from the analysis of lipoprotein compositional data Capurso *et al* (1987) concluded that acipimox did not increase VLDL catabolism but that the reduced LDL mass observed with therapy could be explained by either an increase in catabolism or a reduction in synthesis. Such inconclusive findings on the nature of acipimox's effect on LDL metabolism perhaps prompted the only report of the direct kinetic consequences of acipimox therapy on lipoprotein metabolism, other than that presented here. This was an abstract presenting the results of a series of ten LDL turnovers in patients with familial combined hyperlipidaemia (Angelin, Ericsson, Eriksson, Berglund 1988). This reports the lack of any effect of acipimox on apo-LDL-FCR and is consistent with the findings of the present study and with those of Levy & Langer (1972) who studied the effects of nicotinic acid on apo-LDL metabolism. The latter workers attributed the LDL lowering of nicotinic acid to a marked decrease in the synthetic rate of apo-LDL rather than any increase in catabolism, and went on to suggest that this may be due to a reduction in VLDL synthesis. Although published twenty years ago it appears, by extension of the present study with acipimox back to that of nicotinic acid, that Levy & Langer were indeed correct.

Another way to explain a reduced circulating LDL-cholesterol level would be to propose an inhibitory effect on cholesterol synthesis such as is seen with the statins. However, for acipimox, this does not appear to be the case. Stirling *et al* (1985) studied the effects of acipimox on cholesterol synthesis in human jejunal mucosa. They concluded that this drug had no consistent effect on cholesterol synthesis, which is in accord with findings of Grundy, Mok, Zech & Berman (1981) who studied the metabolism of cholesterol in humans receiving nicotinic acid therapy. They concluded that the drug produced no detectable changes in faecal excretion of cholesterol. More recently, Illingworth *et al* (1991) reported some preliminary findings on urinary mevalonate measurements in niacin treated patients. Using doses of niacin of 1.5-4.5g. d<sup>-1</sup> in patients heterozygous for FH these workers were unable to demonstrate any drug induced effect on urinary mevalonate levels and concluded that niacin had no effect on cholesterol synthesis.

In addition to reducing the total LDL-cholesterol level in the plasma, acipimox therapy also appeared to redistribute the subfractions of LDL, increasing LDL-I and reducing LDL-III. These structural changes have also been observed with acipimox in hypertriglyceridaemia (Franceschini *et al* 1990), where it was shown that on therapy LDL became larger and more buoyant. These workers also were able to demonstrate that LDL isolated from acipimox treated patients had a higher affinity

for the apoB/E receptor *in vitro* than its pre-therapy counterpart. The mechanism for this LDL redistribution is unknown but three mechanisms may be proposed.

Firstly, the inhibitory effect of acipimox on hepatic triglyceride lipase is thought to be important. In hepatic lipase deficiency, IDL and large, buoyant LDL have been shown to predominate in plasma (Demant *et al* 1988, Auwerx *et al* 1989). This suggests that hepatic lipase has an important role in the conversion of IDL to LDL and possibly, by extension, even between LDL subspecies.

Secondly, the redistribution of LDL subfractions may be the result of reduced inter-particle exchange of core lipid. A decrease in the level of triglyceride rich lipoproteins in the circulation would limit the opportunity for the transfer of triglyceride into LDL. The latter would remain relatively cholesteryl ester enriched, as seen here (table 16), and would therefore be less susceptible to lipolysis by hepatic lipase with a consequent increase in particle size and buoyancy.

Thirdly, there is kinetic evidence that various LDL subfractions arise from different ends of the VLDL spectrum. Large VLDL<sub>1</sub> are thought to give rise to small, dense LDL while smaller VLDL<sub>2</sub> are thought to be the potential precursors of larger LDL species (Packard *et al* 1984, Shepherd & Packard 1987). Thus, by causing a shift from VLDL<sub>1</sub> to VLDL<sub>2</sub> synthesis, acipimox may alter the pattern of LDL subfractions. These three mechanisms are, of course, not mutually exclusive but the weight that is given to each will only be resolved by further, specific studies of LDL subfraction kinetics.

In conclusion, the present study provides data in support of the proposed mechanism of acipimox action. There is reduced synthesis of triglyceride rich particles consistent with a reduction in hepatic triglyceride production. There is a marked fall in LDL-cholesterol associated, not with any increase in LDL apoB clearance but with a reduction in synthesis. This reduction in LDL apoB synthesis is shown to be a result of reduced direct synthesis, reduced IDL to LDL transfer consistent with inhibition of hepatic triglyceride lipase, and with a reduction in the overall throughput of VLDL<sub>1</sub> apoB.

## Chapter 6 Ciprofibrate

The science of life is a superb and dazzlingly lighted hall which may only be reached by passing through a long and ghastly kitchen

Claude Bernard 1865

### 6.1. Introduction

The fibrates are a powerful group of drugs that have found an important place in the lipid regulating formulary. While their clinical efficacy is generally accepted their mechanism of action has yet to be fully defined. In particular, their ability to lower plasma LDL-cholesterol levels significantly in patients with hypercholesterolaemia has been a point of great interest and controversy (Gaw & Shepherd 1991)

Studies of the mechanism of action of fibrate therapy, have in the past assumed LDL to be a homogeneous entity (Langer, Strober & Levy 1972, Stewart *et al* 1982). However, more recently it has been demonstrated that LDL exists in the plasma of normal and hyperlipidaemic subjects as a heterogeneous population of particles that may be divided into three subfractions on the basis of size and density (Krauss & Burke 1982, Fisher 1983, Griffin *et al* 1990). In addition to these studies on LDL structure there has been a re-evaluation of the *in vivo* kinetic behaviour of apo-LDL. Classically, analysis of LDL turnover is performed using the model of Matthews (1957), which is based on the assumption that trace-labelled apo-LDL is homogeneous and is cleared from the circulation by a single catabolic mechanism. More recent detailed investigations (Boston *et al* 1982, Foster *et al* 1986) have tested the validity of this assumption and found it to be unwarranted in most subjects. Radioactive products released from the breakdown of trace-labelled



apo-LDL appears quantitatively in the urine. Examination of the daily rate of urinary radioactivity excretion relative to plasma radioactivity levels (U/P ratio) has shown that instead of being constant as predicted by Matthews (1957) this parameter decreases over the period of the turnover study. Therefore, a new model has been postulated to describe apo-LDL metabolism (Boston *et al* 1982, Foster *et al* 1986) in which there are two plasma compartments: Pool A, with a rapid clearance rate and proposed high affinity for apoB/E receptors, and Pool B, with a slower catabolic rate and a proposed reduced level of receptor-mediated catabolism.

In a previous investigation of the mechanism of action of the fibrate, bezafibrate, in type II hyperlipoproteinaemia, Stewart *et al* (1982) ascribed the LDL lowering effect solely to increased clearance of LDL via the apoB/E receptor pathway, as a result of increased receptor expression. In the light of present knowledge of the heterogeneity of LDL it would be pertinent to ask whether this enhanced receptor mediated catabolism of LDL was due to changes in the receptor or the ligand. It is arguable that fibrate therapy may change the LDL subfraction distribution in a way that increases the affinity of LDL particles for the apoB/E receptor. In the present study, this possibility was examined together with detailed studies of the kinetics of apo-LDL using the recently introduced fibric acid derivative, ciprofibrate. This lipid-lowering drug (figure 11) has the longest half-life (Davison *et al* 1975) of the fibrates (approximately 42 h), and its efficacy has been demonstrated in type IIa, IIb, III, and IV hyperlipoproteinaemias (Olsson & Oro 1982, Davignon *et al* 1982). In these studies laboratory safety monitoring has in general not revealed any clinically significant changes, and the drug has been well-tolerated both during short term (Illingworth *et al* 1982) and long term usage (Schifferdecker *et al* 1984).

In the present study, ciprofibrate was used to treat a group of subjects with primary moderate hypercholesterolaemia and normotriglyceridaemia. The results are consistent with the view that the fibrates lower LDL-cholesterol by enhancing receptor mediated clearance of LDL, and that changes in the ligand do indeed contribute to this process.

## 6.2. Protocol

This study of the effects of ciprofibrate on the metabolism of apo-LDL consisted of three phases :

1. Preliminary Screening Period. During this 5 week run in period each patient was screened for cardiological, haematological, hepatic, endocrine, renal, and metabolic disease by routine clinical and laboratory testing.
2. Baseline Study. In this period an LDL turnover study was performed on each patient to serve as a control. Baseline lipid, lipoprotein and lipoprotein subfractions were measured as described above.
3. Active Treatment Period. Immediately after the baseline assessment of apo-LDL metabolism the patients were commenced on ciprofibrate therapy at a dose of 100mg. d<sup>-1</sup>. Patients remained on this dose for 8 weeks. During the final 2 weeks each patient underwent a second turnover investigation while on ciprofibrate therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated.

### 6.3 Subjects

Eleven study patients were selected from the Lipid Clinics at Hairmyres Hospital, East Kilbride and the Victoria Infirmary, Glasgow. All patients had an initial elevated total cholesterol level ( $\geq 7.0$ mmol. L<sup>-1</sup>) despite adherence to a standard lipid lowering diet (Study Group, European Atherosclerosis Society dietary recommendations, 1988) and a triglyceride level less than 2.3mmol. L<sup>-1</sup>, but did not have clinical evidence of FH. Patients were screened for cardiological, haematological, hepatic, endocrine, renal and metabolic disease by routine clinical and laboratory testing to exclude secondary causes of hyperlipidaemia and any illness thought to influence the outcome of the study. Pre-menopausal women were excluded from the study. One patient completed the baseline turnover study and only one week of therapy before asking to be withdrawn from the study. The characteristics of the remaining ten patients are summarised in table 18. Six of the 10 subjects studied were receiving prescribed medications for other clinical conditions. All prescribed medications were continued unchanged throughout the course of the study.

### 6.4. Adverse Events

One patient, described above, asked to discontinue ciprofibrate treatment after 7 days because she reported an exacerbation of her long-standing dyspepsia. Drug was withdrawn and the dyspepsia resolved. The patient did not allow the investigator to rechallenge with ciprofibrate and this event must be recorded as a possible drug-related adverse event. The patient recovered completely and was

Table 18. Summary of Patient Characteristics

Subject	Sex†	Age§	BMI	Apo E Phenotype	Concomitant Drug Therapy	Clinical Diagnoses¶
CIP 01	M	37	24.1	3/3	Ranitidine	Hiatus hernia
CIP 02	M	58	24.4	3/3	Dihydrocodeine, Ketoprofen	Osteoarthritis
CIP 03	F	62	25.0	4/3	Nifedipine, Cimetidine	Angina, peptic ulceration
CIP 04	F	53	27.9	4/3	Nil	Nil
CIP 05	F	61	23.8	3/3	Nil	Angina
CIP 06	M	46	26.2	3/3	Nil	Nil
CIP 07	M	63	25.8	3/3	Nifedipine	Angina
CIP 08	M	63	23.0	3/3	Aspirin	Angina
CIP 09	M	40	27.8	3/3	Nil	Peptic ulceration
CIP 10	M	68	23.4	3/3	Ranitidine, Distalgesic®, Nicorette®	Osteoarthritis, dyspepsia

†Male (M), Female (F). §Ages in years at start of study. || Body mass index (BMI) calculated from weight (kg)/height<sup>2</sup>(m). ¶Current medical problems of subjects at start of study.

discontinued from the study. In the remaining ten patients ciprofibrate was well tolerated. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy.

### 6.5. Lipids and Lipoproteins

Plasma lipid and lipoprotein levels of those patients studied are shown in table 19. Treatment with ciprofibrate significantly affected all the parameters of the lipid profile. Plasma cholesterol was reduced by 17% ( $p < 0.001$ ). This decrement was due, in this group, to a 38% fall in VLDL-cholesterol ( $p < 0.001$ ) and a 22% fall in LDL-cholesterol ( $p < 0.001$ ). There was also a marked fall in plasma triglyceride of 41% ( $p < 0.001$ ) and a rise in HDL-cholesterol of 11% ( $p < 0.02$ ). No significant change in plasma Lp(a) was noted with ciprofibrate therapy. Centrifugal analysis of the subfraction distribution in the HDL and LDL density intervals was performed. No significant change in HDL<sub>2</sub>, HDL<sub>3</sub> or the HDL<sub>2/3</sub> ratio was observed. Figure 45 shows the LDL subfraction profiles obtained upon density gradient fractionation of subjects' plasma. There was some inter-individual variation in the pattern observed, but in most three distinct populations of particles could be distinguished both before and during therapy. The decrement in the major LDL-II species was the most significant change in these subjects. Quantitative analysis revealed that its concentration fell by 28% ( $p < 0.01$ ). LDL-I, the least dense fraction was unaffected by therapy, while the mean level of small, dense LDL-III fell by 31% (table 20). Analysis of the overall composition of LDL (table 21) showed no effect of ciprofibrate therapy, suggesting that the fall in LDL-cholesterol observed was due to a reduction in the number of circulating LDL particles.

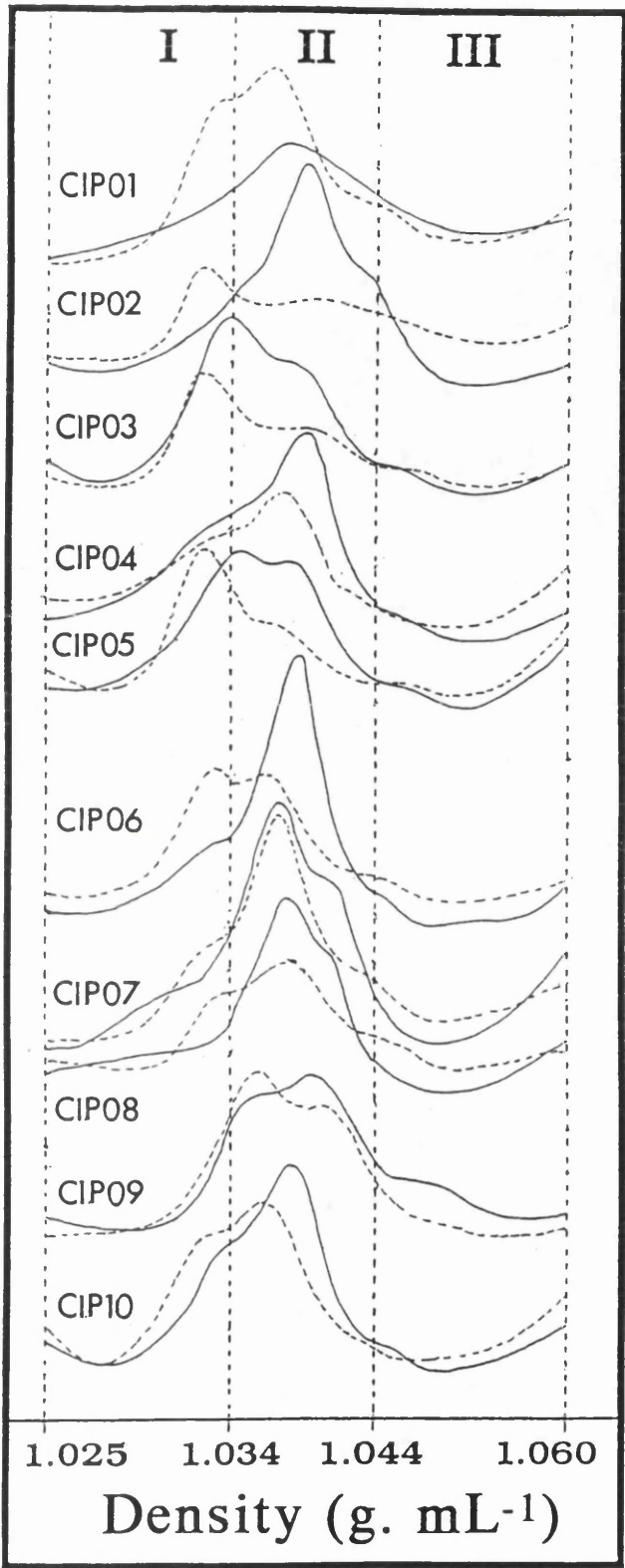
### 6.6. Receptor Mediated and Receptor Independent Apo-LDL Metabolism

The influence of ciprofibrate therapy on apo-LDL metabolism was examined before and on drug using dual-tracer, native and cyclohexanedione-modified apo-LDL turnover studies as described above (section 2.4). In two turnovers, CIP 04B and CIP 07A the decay curves for native and cyclohexanedione-modified LDL could be superimposed suggesting that there may have been some degree of native tracer damage before re-injection into the subject. This may be seen in the plasma, urine and U/P ratio graphs shown in appendix 5. Because of the unsatisfactory technical nature of these turnovers they have been excluded from the analysis of paired data.

Table 19. Lipid and Lipoprotein Changes with Ciprofibrate Therapy

Subject	Cholesterol	Triglyceride	VLDL-C	LDL-C	HDL-C	HDL <sub>2</sub> (mass)	HDL <sub>3</sub> (mass)	Lp(a)
			-----mmol. L <sup>-1</sup> -----	-----	-----	-----mg. dL <sup>-1</sup> -----	-----	mg.dL <sup>-1</sup>
<i>Before Therapy</i>								
CIP 01A	6.5*	1.25	0.64	4.78	1.06	34	175	36
CIP 02A	7.2	1.10	0.60	4.72	1.72	107	392	7
CIP 03A	6.8	1.20	0.65	4.41	1.68	158	239	17
CIP 04A	7.4	1.24	0.51	5.54	1.30	92	229	51
CIP 05A	7.3	1.40	0.70	5.10	1.55	132	229	46
CIP 06A	7.5	1.42	0.75	5.50	1.26	81	219	14
CIP 07A	8.6	1.36	0.81	6.60	1.15	102	209	48
CIP 08A	6.7	2.00	1.02	4.47	1.17	81	290	1
CIP 09A	8.9	2.35	0.88	6.06	1.93	134	361	76
CIP 10A	9.1	1.45	0.69	6.80	1.58	102	348	100
Mean (SEM)	7.6 (0.3)	1.48 (0.12)	0.73 (0.05)	5.40 (0.27)	1.44 (0.09)	102 (11)	269 (23)	40 (10)
<i>On Ciprofibrate</i>								
CIP 01B	6.1	0.66	0.40	4.33	1.40	39	202	35
CIP 02B	5.4	0.68	0.42	2.95	2.00	118	335	8
CIP 03B	5.1	0.61	0.40	2.89	1.79	166	194	15
CIP 04B	6.3	0.79	0.36	4.45	1.44	85	336	62
CIP 05B	6.0	0.69	0.39	3.66	1.93	141	153	51
CIP 06B	6.1	0.88	0.55	4.04	1.53	72	321	15
CIP 07B	7.4	1.18	0.55	5.60	1.25	43	199	43
CIP 08B	5.3	1.10	0.57	3.35	1.38	33	314	18
CIP 09B	8.0	1.18	0.62	5.52	1.82	81	441	72
CIP 10B	7.0	1.07	0.27	5.20	1.48	63	339	67
Mean (SEM)	6.3 (0.3)	0.88 (0.07)	0.45 (0.04)	4.20 (0.32)	1.60 (0.08)	84 (14)	283 (29)	39 (8)
p	<0.001	<0.001	<0.001	<0.001	<0.02	NS	NS	NS

\* Cholesterol, triglyceride VLDL, LDL & HDL-C figures are means of four measurements over the 14 day turnover



**Figure 45.** LDL subfraction profiles at baseline (—) and during ciprofibrate therapy (---) in each of the 10 subjects.

**Table 20.** LDL Subfraction Changes with Ciprofibrate Therapy

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100mL plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
CIP 01A	410	147	227	36
CIP 02A	429	141	202	86
CIP 03A	434	230	181	22
CIP 04A	536	241	269	27
CIP 05A	471	252	195	24
CIP 06A	484	107	328	49
CIP 07A	598	116	341	141
CIP 08A	442	97	253	92
CIP 09A	447	181	223	43
CIP 10A	618	139	451	28
Mean (SEM)	487 (23)	165 (18)	267 (27)	55 (12)
<i>On Ciprofibrate</i>				
CIP 01B	448	161	224	63
CIP 02B	253	114	102	38
CIP 03B	302	143	130	29
CIP 04B	428	192	214	22
CIP 05B	351	224	112	15
CIP 06B	367	176	164	27
CIP 07B	493	116	298	79
CIP 08B	318	108	163	47
CIP 09B	552	129	257	28
CIP 10B	473	180	263	29
Mean (SEM)	399 (30)	154 (12)	193 (22)	38 (6)
p*	<0.02	NS†	<0.01	NS
* Comparison of the means was performed using paired students t-test.				

The kinetic parameters of apo-LDL metabolism are presented in table 22. Ciprofibrate was associated with a 17% increase in the overall FCR of apo-LDL ( $p<0.002$ ). This change was apparently responsible for a 17% fall in the plasma concentration of apo-LDL ( $p<0.05$ ) as there was no significant effect of drug therapy on total apo-LDL synthetic rate. When LDL catabolism was divided into receptor-mediated versus receptor independent pathways, it was observed that the FCR of the former was increased by 34% ( $p<0.03$ ) while the latter was unchanged. Although the rate at which LDL was cleared by the high affinity receptor pathway

**Table 21.** Effects of Ciprofibrate on LDL Composition.

Subject	Free Cholesterol	Cholesteryl Ester	Triglyceride	Phospholipid	Protein
-----g/100g-----					
<i>Before Therapy</i>					
CIP 01A	13.1	34.1	5.2	21.9	25.6
CIP 02A	14.5	36.9	3.5	20.0	25.2
CIP 03A	12.6	32.1	6.3	19.8	29.3
CIP 04A	12.8	34.1	7.5	20.8	24.8
CIP 05A	12.2	33.9	5.5	20.2	28.2
CIP 06A	11.8	35.1	5.1	19.0	29.0
CIP 07A	10.3	40.0	4.6	16.7	28.6
CIP 08A	10.6	36.1	8.4	17.8	27.3
CIP 09A	12.9	36.7	4.8	21.1	24.6
CIP 10A	13.4	13.4	6.1	20.2	25.9
Mean (SEM)	12.4 (0.4)	33.2 (2.3)	5.7 (0.5)	19.8 (0.5)	26.9 (0.6)
<i>On Ciprofibrate</i>					
CIP 01B	9.9	33.7	4.6	18.4	28.6
CIP 02B	11.0	39.2	6.1	21.9	21.9
CIP 03B	10.5	37.0	6.0	19.8	26.7
CIP 04B	14.6	31.4	6.8	21.3	25.9
CIP 05B	11.2	37.0	5.6	20.6	25.6
CIP 06B	13.1	38.4	5.8	16.6	26.0
CIP 07B	10.7	39.4	5.0	19.9	25.1
CIP 08B	15.0	33.5	5.5	18.4	27.7
CIP 09B	6.7	40.6	8.4	18.0	26.0
CIP 10B	12.9	32.3	9.3	23.0	24.5
Mean (SEM)	11.6 (0.8)	36.3 (1.0)	6.3 (0.5)	19.8 (0.6)	25.8 (0.6)

increased with ciprofibrate therapy, there were no significant change in the absolute amount of apo-LDL cleared by either pathway.

Examination of the plasma decay curves in conjunction with the daily urinary radioactivity excretion rates (shown for subject CIP 08 in figures 46-51) revealed that the drug was perturbing LDL metabolism in a complex fashion. During the control turnover, the amount of radioactivity excreted into the urine was virtually a fixed proportion of the radioactivity present in the plasma. The calculated U/P ratio (figure 48), a daily index of catabolic potential, fell only slightly from a peak value of 0.37 at 2-3 days post-injection to 0.30, by days 10-12. On ciprofibrate, however, the relationship between the urine and plasma decay curves was



Table 22. Effects of Ciprofibrate Therapy on Apo-LDL Metabolism I

Subject	Apo-LDL mg. dL <sup>-1</sup>	FCR pools. d <sup>-1</sup>	FCR CHD-LDL pools. d <sup>-1</sup>	FCR RM pools. d <sup>-1</sup>	Synthetic Rate* mg.kg <sup>-1</sup> .d <sup>-1</sup>	Absolute Catabolic Rate† Recep Ind Recep Med mg.kg <sup>-1</sup> .d <sup>-1</sup>
<i>Before Therapy</i>						
CIP 01A	105	0.30	0.16	0.14	12.5	6.7
CIP 02A	108	0.27	0.13	0.14	11.7	5.7
CIP 03A	126	0.28	0.14	0.14	14.1	7.2
CIP 04A	101	0.21	0.12	0.10	8.6	4.7
CIP 05A	133	0.30	0.17	0.13	16.1	9.2
CIP 06A	141	0.31	0.16	0.15	17.3	9.1
CIP 07A	171	-	0.16	-	-	10.7
CIP 08A	121	0.32	0.16	0.16	15.5	7.9
CIP 09A	110	0.15	0.13	0.02	6.8	5.8
CIP 10A	160	0.20	0.12	0.07	12.5	7.9
Mean (SEM)	128 (8)	0.26 (0.02)	0.15 (0.01)	0.12 (0.02)	12.8 (1.2)	7.5 (0.6)
<i>On Ciprofibrate</i>						
CIP 01B	128	0.33	0.17	0.17	17.1	8.6
CIP 02B	56	0.26	0.14	0.12	5.9	3.1
CIP 03B	80	0.33	0.14	0.19	10.7	4.6
CIP 04B	111	-	0.13	-	-	5.6
CIP 05B	90	0.27	0.13	0.14	9.8	4.7
CIP 06B	96	0.30	0.13	0.16	11.3	5.1
CIP 07B	124	0.31	0.15	0.16	15.3	6.3
CIP 08B	88	0.37	0.17	0.20	13.0	7.6
CIP 09B	120	0.30	0.17	0.12	14.2	5.9
CIP 10B	165	0.26	0.15	0.12	17.2	8.3
Mean (SEM)	106 (10)	0.30 (0.01)	0.15 (0.01)	0.15 (0.01)	12.7 (1.2)	6.3 (0.7)
p	<0.05	<0.002	NS	<0.03	NS	NS

\* synthetic rate is the product of the fractional catabolic rate (pools. d<sup>-1</sup>) and the pool of apo-LDL (mg), divided by body weight (kg).

† 'receptor independent' apo-LDL clearance is calculated from the FCR of cyclohexanedione modified LDL multiplied by the apo-LDL pool size (mg) divided by the body weight (kg). The 'receptor-mediated' component is the difference between receptor-mediated and total apo-LDL clearance. The latter is the product of the FCR of native LDL and the apo-LDL pool size, and under steady state conditions this equals the value for the apo-LDL synthetic rate.

substantially altered (figure 51). In the first 5-6 days of the turnover, the plasma curve showed a more rapid decay than in the control phase. This change was due to enhanced catabolism rather than increased extravascular exchange, because there was a concomitant rise in urinary radioactivity excretion. The U/P ratio in this subject (figure 51) was initially high (0.60) and showed a substantial fall over the first 5-6 days to the same final value as in the control phase (0.30).

### 6.7. Mathematical Modelling of Apo-LDL Metabolism

The effects of ciprofibrate on apo-LDL metabolism were further studied by subjecting the plasma and urine radioactivity obtained from each patient to more detailed analysis using multicompartmental modelling techniques described above. Analysis of this data using the two plasma pool model depicted in figure 15 generated the results shown in table 23 and summarised in figures 52 & 53. Again, those turnovers that were technically unsatisfactory viz CIP 04B and CIP 07A have been excluded from the analysis. In the control phase, pool A (the more rapid cleared species) accounted for approximately one third of the total circulating mass of apo-LDL. Its mean synthetic rate was 749 mg. d<sup>-1</sup> and it had a fractional catabolic rate of 0.69 pools. d<sup>-1</sup>. The mean mass of apo-LDL in pool B was approximately 2000mg and in most subjects this was the major apo-LDL component. Its FCR was a third and its synthetic rate a half of that in pool A. There was a trend for ciprofibrate therapy, to increase the synthesis of pool A and at the same time decrease that of pool B. The overall result of these balanced changes was no difference in the the synthesis of total apo-LDL as shown in table 22. The fractional clearance rate of pool B was unaltered by therapy, but that of pool A rose in 7 of the 8 subjects studied. The net effect of these changes was that while the computed mass of apo-LDL in pool A remained constant that in pool B was reduced by 25%.

### 6.8. Discussion

The results of this study give valuable information not only on the effects of the lipid lowering drug, ciprofibrate, but also on the underlying metabolic heterogeneity evident in LDL. As such the following discussion has been divided in two parts to allow both facets to be fully explored.

Figure 46. Native LDL

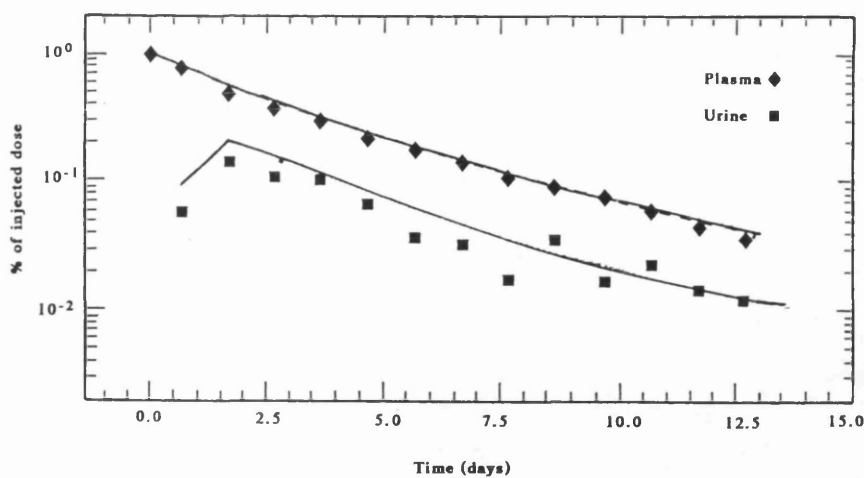


Figure 47. Cyclohexanedione Modified LDL

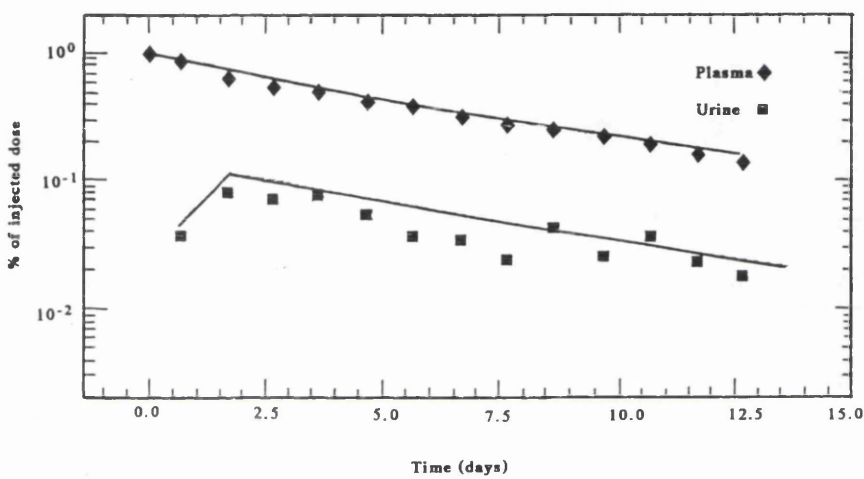
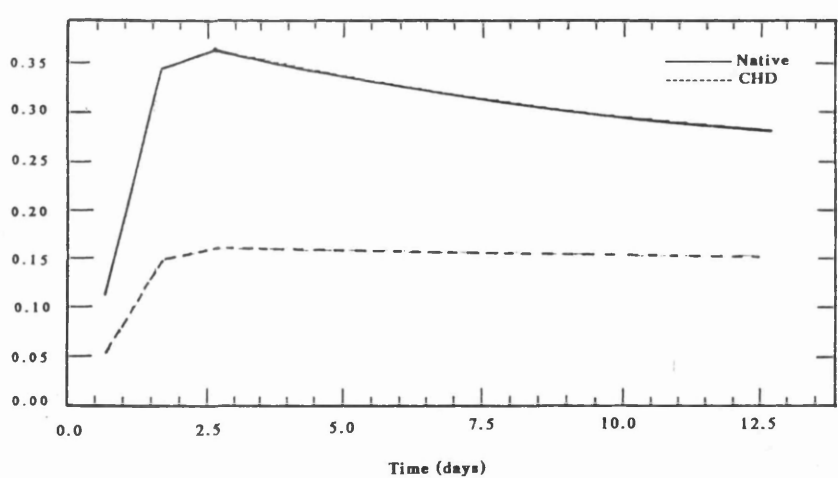


Figure 48. U/P Ratios



**Figure 46-48.** Baseline. Plasma and urine radioactivity decay curves following injection of [ $^{125}\text{I}$ ]-native apo-LDL and [ $^{131}\text{I}$ ]-cyclohexanedione-modified apo-LDL. Subjects received a bolus dose of apo-LDL tracers and daily plasma samples were taken over 14 days. Urine was collected in each 24 h period and the urinary radioactivity excretion examined. The urine/plasma ratio was calculated as the urinary loss divided by the plasma radioactivity at the beginning of the 24 h collection period. Results are shown for subject CIP 08 in the control phase.

Figure 49. Native LDL

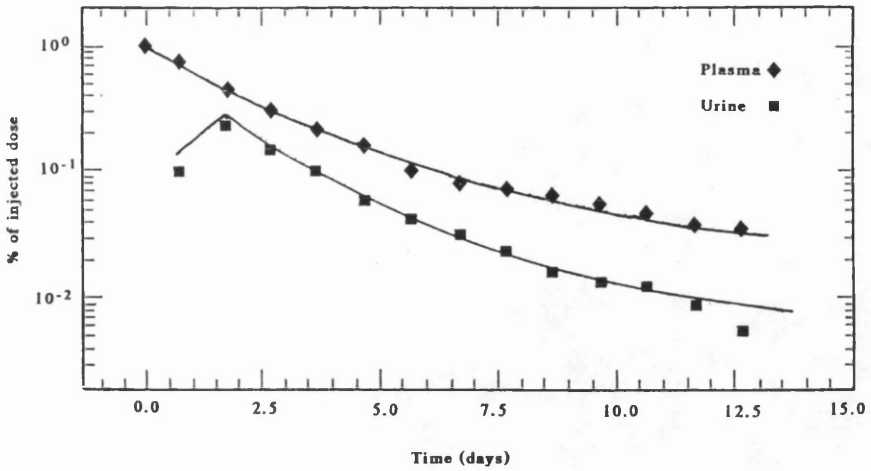


Figure 50. Cyclohexanedione Modified LDL

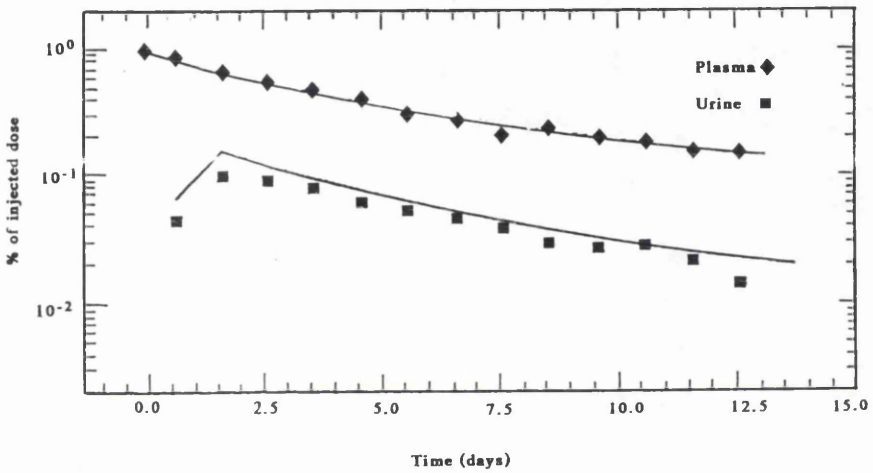
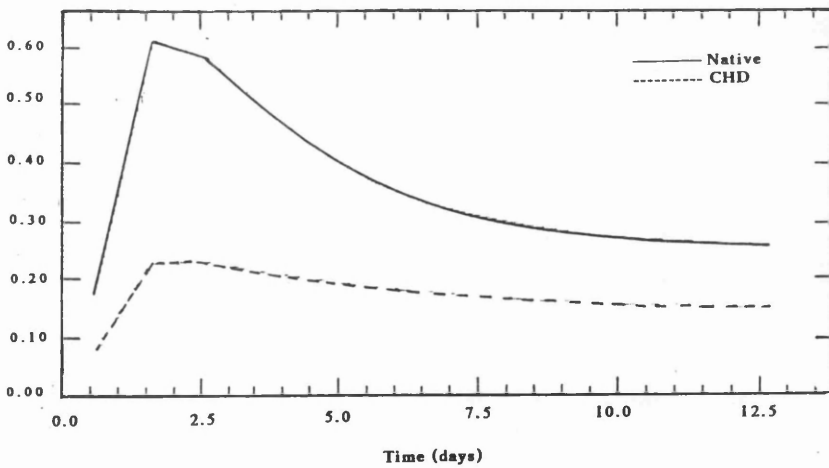
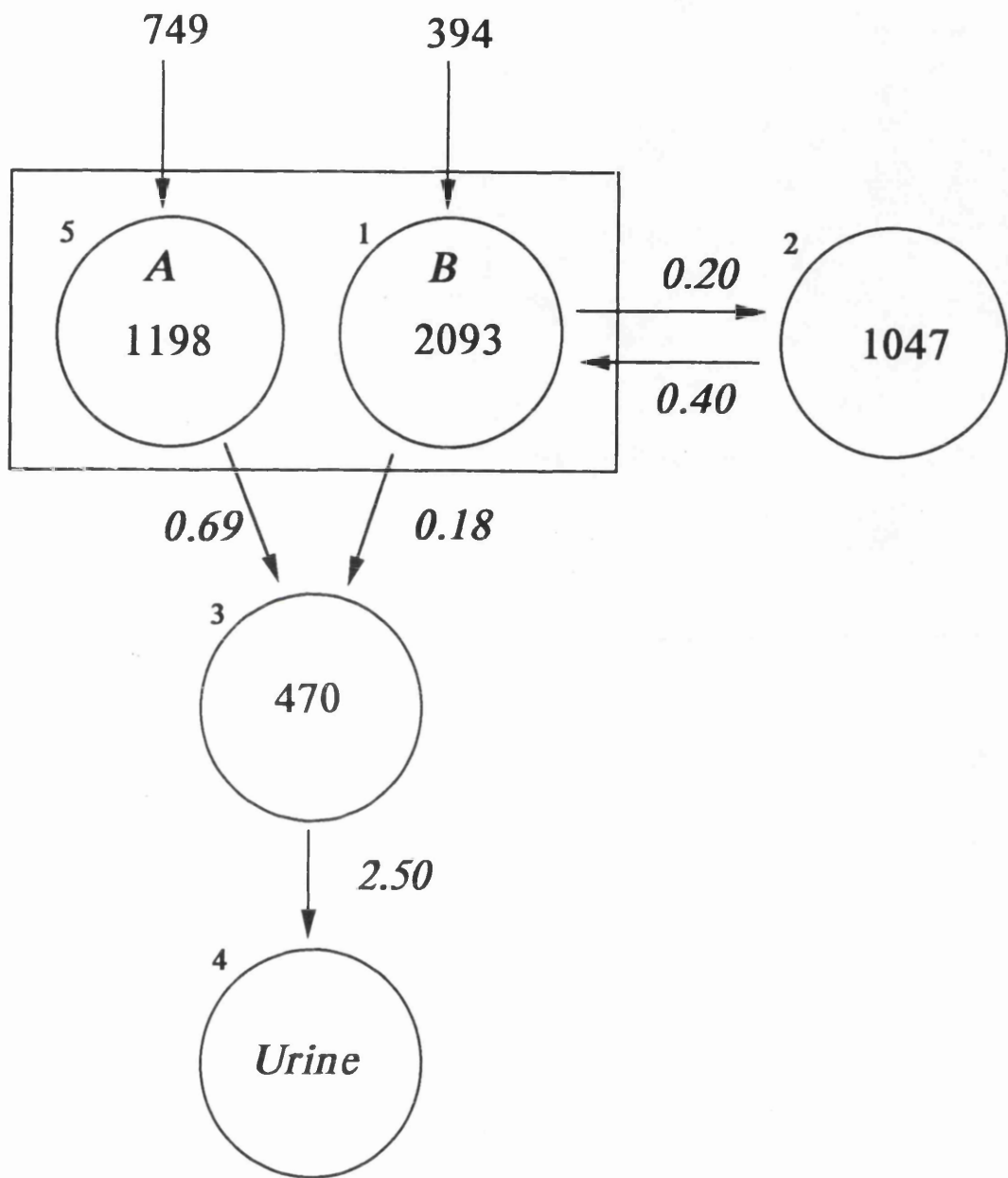


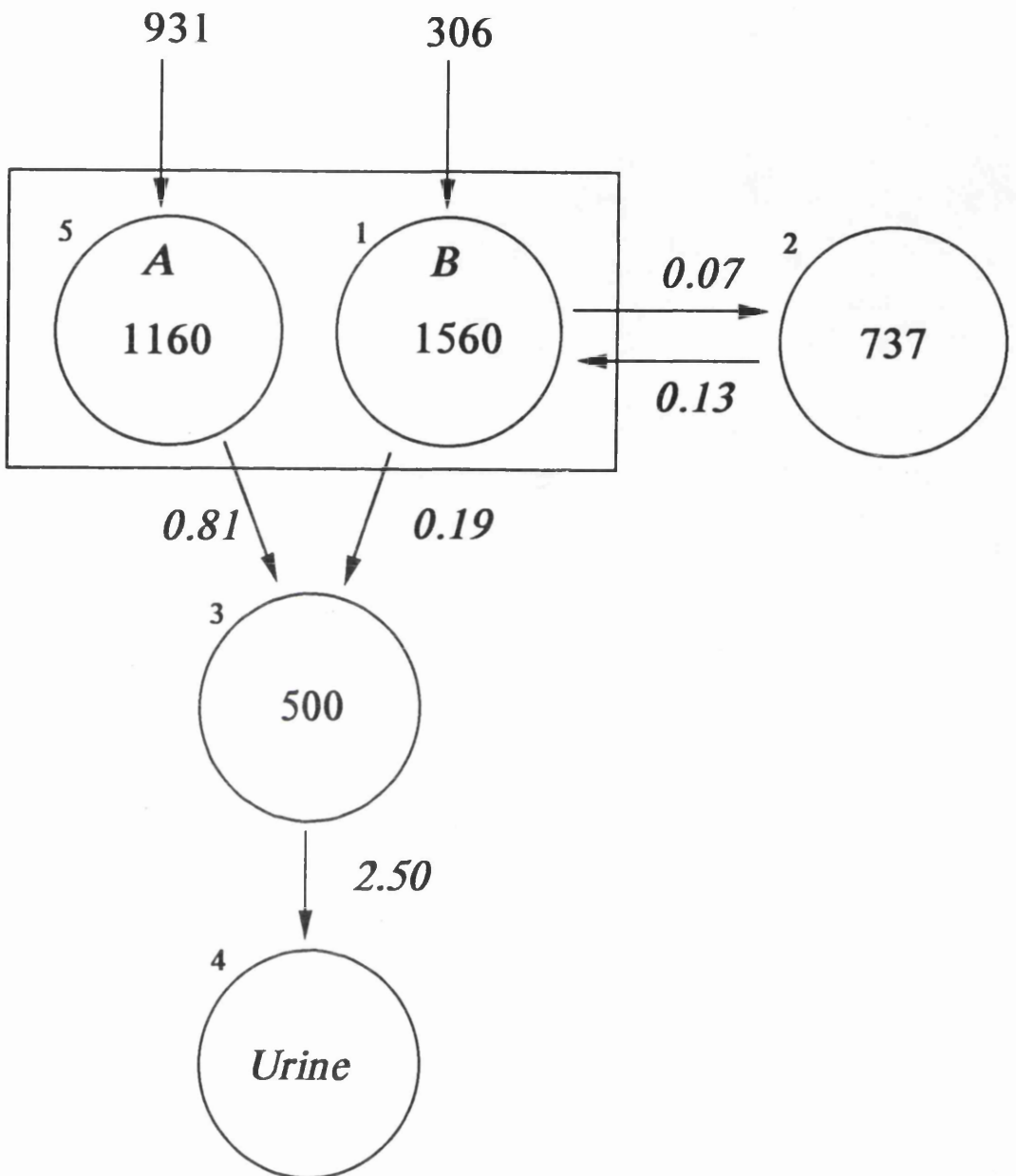
Figure 51. U/P Ratios



**Figure 49-51.** On ciprofibrate. Plasma and urine radioactivity decay curves following injection of [ $^{125}\text{I}$ ]-native apo-LDL and [ $^{131}\text{I}$ ]-cyclohexanedione-modified apo-LDL. Subjects received a bolus dose of apo-LDL tracers and daily plasma samples were taken over 14 days. Urine was collected in each 24 h period and the urinary radioactivity excretion examined. The urine/plasma ratio was calculated as the urinary loss divided by the plasma radioactivity at the beginning of the 24 h collection period. Results are shown for subject CIP 08 on ciprofibrate.



**Figure 52.** Summary of apo-LDL metabolism at baseline in eight subjects. Numbers in italics on arrows represent fractional transfer rates in  $\text{pools} \cdot \text{d}^{-1}$ . Numbers in circles represent mean apo-LDL mass in mg.



**Figure 53.** Summary of the effects of ciprofibrate therapy on apo-LDL metabolism in eight subjects. Numbers in italics on arrows represent fractional transfer rates in pools.  $\text{d}^{-1}$ . Numbers in circles represent mean apo-LDL mass in mg.

Table 23. Effects of Ciprofibrate Therapy on Apo-LDL Metabolism II

Subject	Apo-LDL Pool A*			Apo-LDL Pool B†		
	Computed Mass mg	FCR pools. d <sup>-1</sup>	SR‡ mg.d <sup>-1</sup>	Computed Mass mg	FCR pools. d <sup>-1</sup>	SR mg. d <sup>-1</sup>
<i>Before Therapy</i>						
CIP 01A	1021	1.31	1338	2192	0.22	482
CIP 02A	430	0.79	340	2637	0.23	607
CIP 03A	1442	0.44	634	1033	0.17	176
CIP 05A	370	0.47	174	2524	0.21	530
CIP 06A	2824	0.46	1299	1790	0.19	340
CIP 08A	590	0.53	313	2710	0.24	650
CIP 09A	1879	0.46	864	1531	0.04	61
CIP 10A	1033	1.04	1074	2327	0.13	303
Mean (SEM)	1198 (295)	0.69 (0.12)	749 (161)	2093 (210)	0.18 (0.02)	394 (74)
<i>On Ciprofibrate</i>						
CIP 01B	1509	1.34	2022	2011	0.21	422
CIP 02B	645	1.00	645	938	0.16	150
CIP 03B	869	0.71	617	686	0.17	117
CIP 05B	933	0.57	532	1306	0.18	235
CIP 06B	1001	0.71	711	2112	0.22	465
CIP 08B	1252	0.73	914	1148	0.23	264
CIP 09B	1677	0.71	1191	2288	0.19	435
CIP 10B	1394	0.70	976	1990	0.16	318
Mean (SEM)	1160 (125)	0.81 (0.09)	931 (172)	1560 (216)	0.19 (0.01)	306 (47)
P	NS	NS	NS	NS	NS	NS

\* Pool A, computed mass (M5); Fractional catabolic rate L(3,5), † Pool B, computed mass (M1); Fractional catabolic rate L(3,1), ‡SR=synthetic rate.

### 6.8.1. Effects of Ciprofibrate.

In the present study, using ciprofibrate (100mg. d<sup>-1</sup>), highly significant changes in all the parameters of the  $\beta$ -quantification were achieved. The comparative efficacies of the fibrates in type IIa hyperlipoproteinaemia are summarized in table 24, where it can be seen that the percentage changes obtained with ciprofibrate in this study are consistent, and in many instances better than, the changes obtained with similar drugs in similar patients.

In order to discuss the full implications of the findings in the present study it is necessary to review more fully the known mechanisms of action of the fibrates.

The primary action of the fibrates is to reduce plasma levels of triglyceride rich lipoproteins. This action is thought to have two main mechanisms:

- 1) decreased hepatic triglyceride synthesis, which is secondary to reduced peripheral lipolysis and diminished free fatty acid flux back to the liver.
- 2) enhanced very low density lipoprotein (VLDL) catabolism, due to stimulated lipoprotein lipase activity and consequently increased intravascular VLDL triglyceride lipolysis.

This latter mechanism is most recently documented by Chan (1989) and Vessby & Lithell (1990), who reported increased post heparin lipoprotein lipase activity with gemfibrozil and bezafibrate treatment respectively.

The fibrate induced increase in lipoprotein lipase activity noted above offers an explanation for the fall in post prandial lipaemia seen in both normo- and hypercholesterolaemic patients treated with fenofibrate (Simpson *et al* 1990). This improvement in chylomicron metabolism was associated with a highly significant increase in post heparin lipoprotein lipase activity (37%,  $p < 0.001$ ), and only a marginal rise in that of hepatic lipase. Such an effect of the fibrates may be very important contribution to their anti-atherogenic potential, as post-prandial lipaemia is considered by many to be an additional coronary heart disease risk factor (Zilversmit 1979). The presence of CHD has also been associated with prolonged and exaggerated hypertriglyceridaemia following a fat load. Such abnormal clearance of chylomicrons and their remnants seen in patients with CHD is corrected by fibrate therapy (Simpson *et al* 1990). The fibrates, therefore, reduce levels of triglyceride-rich lipoproteins in a consistent and readily explicable fashion.



Table 24. Comparative Effects of the Fibrates in Type IIa Hyperlipoproteinemia

Drug	Reference	Daily Dose	n	% Change on Therapy				
				Cholesterol	Triglyceride	LDL-C	HDL-C	VLDL-C
<i>Clofibrate</i>	Grundy <i>et al</i> 1972	2g	5	-19	-26	*	*	*
	Levy <i>et al</i> 1972	2g	10	-7	-2	*	*	*
<i>Bezafibrate</i>	Stewart <i>et al</i> 1982	600 mg	4	-16	-35	-18	+2	-42
	Olsson <i>et al</i> 1985	600 mg	8	-16	-31	-22	+10	-67
	Gavish <i>et al</i> 1986	600 mg	12	-19	-25	-23	+11	*
	Curtis <i>et al</i> 1988	600 mg	16	-16	-18	-19	-10	-25
<i>Gemfibrozil</i>	Manninen <i>et al</i> 1988	1200 mg	1293	-11	-38	-13	+11	*
<i>Fenofibrate</i>	Canzler <i>et al</i> 1980	300 mg	10	-21	-16	*	*	*
	Lehtonen <i>et al</i> 1981	300-600 mg	16	-20	-27	-20	+15	*
	Rouffy <i>et al</i> 1985	300 mg	21	-20	-16	-25	+14	*
	Rouffy <i>et al</i> 1985	400 mg	21	-28	-18	-33	+11	*
<i>Ciprofibrate</i>	Olsson <i>et al</i> 1982	100 mg	6	-25	-31	-28	+11	-49
	Illingworth <i>et al</i> 1982	50 mg	7	-11	-22	-13	+8	*
	Illingworth <i>et al</i> 1982	100 mg	9	-20	-30	-24	+10	*
	Rouffy <i>et al</i> 1985	100 mg	20	-21	-21	-20	+20	*
	Dairou <i>et al</i> 1986	100 mg	2418	-23	-21	-30	+5	*

\* Not Reported

The action of the fibrates on LDL levels are variable depending on the initial LDL-cholesterol value. In hypercholesterolaemia where LDL levels are high, the fibrates cause a decrease in LDL apoB, while in hypertriglyceridaemia, which is commonly associated with low plasma LDL concentrations, fibrate treatment will raise the latter (Shepherd *et al* 1984b, 1985). This differential in LDL response dependent on initial LDL and triglyceride levels has most recently been reported by Manttari *et al* (1990). The FCR of LDL apoB, in response to fibrate therapy, has previously been shown to increase in hypercholesterolaemia (Stewart *et al* 1982) and decrease in hypertriglyceridaemia (Shepherd *et al* 1984b) while apo-LDL synthetic rates in both groups remain unchanged. This apparent paradox is resolved when these changes are regarded as normalization phenomena: in hypercholesterolaemia an initially low FCR is increased to normal and in hypertriglyceridaemia an initially high FCR is reduced to normal.

The kinetic results of the present study are very similar both to those reported for bezafibrate (Stewart *et al* 1982) and unpublished findings for fenofibrate (Caslake, Packard, Gaw *et al*, submitted manuscript 1992). In both these studies the FCR of apo-LDL increased during fibrate therapy to account for the reduction seen in plasma LDL-cholesterol. In this study, a more detailed kinetic analysis of plasma and urine data was employed, and it may be concluded that the changes in LDL metabolism were due to a combination of two main effects: a shift in synthesis, favouring the formation of the rapidly metabolised species (pool A) and an increase in its rate of elimination. In a previous study of normal individuals, Caslake *et al* (1992) observed that  $L(3,5)$  (the elimination rate constant of pool A) was much more variable than  $L(3,1)$  (the elimination rate constant of pool B) and that when the turnover of native and cyclohexanedione-modified apo-LDL were modelled together, the former pathway was more closely associated with receptor activity than the latter. In light of these findings, the present increase in  $L(3,5)$  associated with ciprofibrate therapy, may be interpreted as an indication that apoB/E receptor activity has been stimulated, perhaps secondary to a fibrate induced suppression of hepatic cholesterol synthesis (Berndt, Gaumert & Still 1978, Stewart *et al* 1982). However, the principal mechanism for the 22% fall in plasma LDL-cholesterol seen in table II was that on therapy the LDL particles formed were better ligands for the apoB/E receptor, as reflected in the relative increase in pool A material. This may have arisen because ciprofibrate caused a reduction in triglyceride relative to apoB synthesis in the liver and therefore promoted the secretion of smaller VLDL precursor particles. These, on the basis of previous studies (Demant *et al* 1991a, Griffin *et al* 1990) are thought to generate receptor-active LDL as the end-product of

intravascular lipolysis. Further evidence to support this hypothesis, comes from studies of type III (Packard *et al* 1986) and type IV (Shepherd *et al* 1984b) hyperlipoproteinaemic subjects, which show that fibrate therapy causes a reduction in the synthesis of large triglyceride-rich VLDL (VLDL<sub>1</sub> Sf 60-400).

### 6.8.2. Use of a Two Compartment Model for LDL Metabolism

Despite the fact that LDL structure, function and metabolism have been studied extensively for twenty years there is still much to be learned about the pathophysiology of this lipoprotein and its role in atherogenesis. Recognition that LDL is structurally and metabolically heterogeneous has been a significant step forward. Foster *et al* (1986) described a number of two-compartment models of apo-LDL metabolism that were all capable of accommodating urine and plasma radioactivity data. One of these, shown in figure 15, was adopted for the present as well as previous studies (Caslake *et al* 1992, Caslake *et al* submitted manuscript 1992) because it was compatible with the LDL sub-system in the much larger model describing the VLDL-IDL-LDL delipidation cascade shown in figure 14 and used in the studies reported in chapters 3,4,5,7 & 8). In these studies, as well as in other published work (Packard *et al* 1984, Demant *et al* 1991a) the presence of parallel pathways of LDL synthesis and catabolism have been demonstrated. Small VLDL (VLDL<sub>2</sub> Sf 20-60) gave rise to a species of LDL that was cleared rapidly from the circulation with an FCR of 0.3-0.5 pools. d<sup>-1</sup> similar to that seen in 'pool A'. Large VLDL (VLDL<sub>1</sub> Sf 60-400), on the other hand, when lipolysed produced LDL that was removed more slowly with an FCR of approximately 0.2 pools. d<sup>-1</sup>, equivalent to that seen for 'pool B' material.

The model shown in figure 15 has been used to provide an explanation for the relationship between plasma triglyceride and LDL metabolism in normolipaemic subjects (Caslake *et al* 1992). In this work it was observed that as plasma triglyceride rose, an increasing proportion of apo-LDL was located in the slowly metabolised pool B. This component was present at about 400mg of circulating mass in those with low plasma triglyceride levels (<1.0 mmol.L<sup>-1</sup>), but became predominant (1500mg circulating mass) at high-normal plasma triglyceride values (1.5-2.0mmol.L<sup>-1</sup>). Apo-LDL in pool A was relatively invariant accounting for approximately 1000mg in most subjects. Application of the model to the present study provided an intuitively acceptable mechanism for the action of ciprofibrate.

The model has certain features that appear to have physiological significance. First, L(3,5) in figure 15 is a key variable in controlling the rate of LDL catabolism. Removal by this route is linked to the activity of the high affinity apoB/E receptor pathway (Caslake *et al* 1992). Second, overall LDL clearance is also influenced by the distribution of mass between pools A and B. This in turn is dependent on their relative synthetic rates and possibly as described above on the nature of the VLDL precursors made by the liver. Third, the FCR of pool B is relatively constant in most subjects. This is a reflection of the observation that in normals (Caslake *et al* 1992) and in the hypercholesterolaemic subjects studied here (figures 46 & 49) parallel terminal exponentials appear after 8-10 days in both plasma and urine radioactivity curves. These give a U/P ratio of 0.2-0.3. The mass of pool B appears to regulate the overall plasma LDL level and hence its atherogenic potential (table 23). Clearly, it is of interest to determine the nature of the LDL that contains this slowly catabolised apo-LDL. Since its concentration is directly related to the plasma triglyceride level in the same way as small, dense LDL in studies from Austin *et al* (1988) and Griffin *et al* (1990) it was initially speculated that the apo-LDL in pool B resided in the LDL-III species. However, on quantitation of the latter (table 20) it is clear that the pool B mass exceeds that of the apolipoprotein in LDL-III alone. It may be proposed that pool B consists of the apoB in LDL-III and a portion of that in LDL-II. This hypothesis is supported by two sets of observations. Firstly, in the present study, the 25% fall in pool B associated with ciprofibrate therapy was accompanied by a significant redistribution of LDL subfractions with the greatest falls in LDL-II (28%,  $p < 0.01$ ) and LDL-III (31%). Secondly, Shepherd *et al* (1991) reported that in patients who are normotriglyceridaemic, yet hypercholesterolaemic, fibrate therapy lowers the total LDL mass and that this is predominantly due to a significant decrease in the LDL subfraction of mid-density (LDL-II). These workers went on to report that the changes in the LDL subfractions of lower density (LDL-I) and higher density (LDL-III) are inversely related, the most consistent finding, during fibrate therapy, being an increase in LDL-I and a decrease in LDL-III.

## 6.9. Conclusion

In conclusion, ciprofibrate therapy in primary, moderate hypercholesterolaemia is associated with highly significant and clinically relevant changes in the lipoprotein profile, associated with changes in the kinetic parameters describing the metabolism of apo-LDL. In the latter, there are changes predominantly in the high-affinity receptor mediated clearance of LDL, which may be explained by a drug induced

shift in the synthesis of LDL towards a species more readily removed by the apoB/E receptor. These data do not exclude the possibility of a fibrate induced increase in expression of the apoB/E receptor in addition to these observed alterations in the ligand.

## Chapter 7 *Combination I Colestipol & Simvastatin*

The whole of science is nothing more than a refinement of everyday thinking.

Albert Einstein

### 7.1 Introduction

Combined drug therapy for hyperlipidaemia is being used increasingly. There are a number of reasons for using combined therapy and these are described in section 1.6.7. Two groups of patients may particularly benefit from this strategy viz. those heterozygous FH patients, who do not respond adequately to monotherapy (Miettinen 1990), and patients with established CHD, where inhibition of the progression of atherosclerosis, and perhaps the induction of its regression are possible with profound lipid lowering (Blankenhorn *et al* 1987, Brown *et al* 1990).

Therapy with a bile acid sequestrant resin plus an HMG CoA-reductase inhibitor has been shown to render between 60-70% of FH heterozygotes normocholesterolaemic (Weisweiler 1988, Leren *et al* 1988, Illingworth & Bacon 1989), and to influence beneficially both progression and regression of atherosclerotic lesions (Brown *et al* 1990).

The first investigation into the effects of such a combination of drugs on lipoprotein metabolism was reported by Bilheimer *et al* (1983). These workers studied the apo-LDL kinetics in a male FH heterozygote, before and during lovastatin and

colestipol therapy. The marked fall in LDL-cholesterol that they observed was solely attributed to an increase in apo-LDL clearance. Grundy, Vega & Bilheimer (1985) went on to investigate the same combination of drugs in a larger group of FH heterozygotes. They again showed the regimen to be highly effective in reducing LDL-cholesterol (52%) but on this occasion they attributed the fall to a combination of increased clearance and reduced production of apo-LDL. A more recent study (Vega, East & Grundy 1989) of FH heterozygotes, and the same combination drug therapy of lovastatin and colestipol, reported that the fall in LDL-cholesterol could be attributed only to enhanced LDL apoB clearance.

These equivocal results are further confounded when another series of experiments is considered. Studies in miniature pigs (Huff *et al* 1985, Huff & Telford 1989) using lovastatin and cholestyramine have shown that the combined drug regimen, in this species, causes a marked reduction in LDL direct synthesis and that VLDL-derived LDL synthesis is unaffected. Furthermore, Huff & Telford (1989) reported an increase in VLDL clearance, suggesting that although no obvious increase in LDL apoB FCR was observed these drugs may still enhance apoB/E receptor activity resulting in the removal of LDL precursor lipoproteins from the circulation. The only published study designed to examine the effects of this drug combination in the same type of patients studied here (i.e. primary moderate hypercholesterolaemia) was that reported by Vega & Grundy (1987). These workers studied a group of 10 subjects with an initial plasma total cholesterol  $>6.5\text{mmol. L}^{-1}$  and normal triglyceride levels. While there was some heterogeneity of response they concluded that the significant reduction observed in LDL-cholesterol (48%) was due to combination of three factors: i) a 27% fall in the production rate of apo-LDL; ii) a 20% rise in apo-LDL FCR; and iii) a 15% depletion in the cholesterol content of LDL particles.

The aim of the present study was to examine in detail the mechanism of action of combined bile acid sequestrant resin and HMG CoA reductase inhibitor therapy in patients with primary moderate hypercholesterolaemia. The patients studied all had established coronary heart disease and were chosen as such to ensure that the potentially large alterations expected in their lipid and lipoprotein profiles would be ethically acceptable. The results confirmed the marked reduction in LDL-cholesterol with combined therapy seen in other studies and studies of the metabolism of the apoB containing lipoproteins also revealed marked changes in IDL and LDL apoB kinetics, which may be explained by enhanced apoB/E receptor function.

## 7.2. Protocol

This extension of the study described in chapter 4 was designed to examine the influence of colestipol and simvastatin on the metabolism of apoB containing particles. The preliminary screening period and baseline study period were as described in chapter 4. On completion of the second VLDL turnover study, the patients were commenced on simvastatin 20mg nocte in addition to their resin therapy, colestipol 20g. d<sup>-1</sup>. Patients remained on these doses for 10 weeks. During the final 2 weeks each patient underwent a third and final VLDL turnover investigation while on combined colestipol and simvastatin therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated.

## 7.3 Subjects

The eight study patients were those described in chapter 4 and their characteristics are summarised in table 8. As before all concomitant medications were continued unchanged throughout this second study phase.

## 7.4 Adverse Events

Combination therapy with colestipol and simvastatin was reasonably well tolerated by all subjects participating in the study. Only one subject, COL 08 complained of any adverse side effects. This patient experienced severe constipation after the addition of simvastatin to his regimen, but this resolved with increased fluid intake and did not necessitate any further intervention or interruption of therapy. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy. Similarly, ophthalmological examination with a slit lamp failed to detect any alteration from baseline in the lens of each subject.

## 7.5 Lipids and Lipoproteins

Baseline plasma lipid and lipoprotein levels of those patients studied are shown in chapter 4, and their levels after combination therapy are displayed in table 25. Combined treatment reduced plasma cholesterol by 38% ( $p < 0.001$ ). This decrement was due in this group to a 52% fall in LDL-cholesterol ( $p < 0.001$ ), while VLDL-cholesterol remained unchanged. Triglyceride was not significantly affected



Table 2.5. Lipid and Lipoprotein Changes with Combined Colestipol and Simvastatin Therapy

Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	HDL <sub>2</sub> (mass)	HDL <sub>3</sub> (mass)	Lp(a)
			-----mmol. L <sup>-1</sup> -----		-----	-----mg. dL <sup>-1</sup> -----		mg. dL <sup>-1</sup>
<i>Before Therapy</i>								
Mean	6.57	1.87	0.84	4.56	1.10	49	225	20
(SEM)	(0.17)	(0.14)	(0.07)	(0.20)	(0.02)	(11)	(8)	(11)
<i>On Colestipol and Simvastatin</i>								
COL 01C	3.33	1.22	0.42	1.80	1.12	60	193	4
COL 02C	4.32	1.82	0.48	2.55	1.28	55	243	13
COL 03C	4.37	2.12	0.77	2.50	1.10	79	363	105
COL 04C	4.80	1.97	0.85	2.78	1.17	61	379	13
COL 05C	3.83	0.97	0.40	1.95	1.48	89	240	4
COL 06C	3.78	1.52	0.55	2.00	1.23	16	233	27
COL 07C	3.83	1.12	0.67	1.97	1.20	57	290	14
COL 08C	4.37	3.03	1.25	2.00	1.12	83	217	9
Mean	4.08	1.72	0.67	2.19	1.21	63	270	24
(SEM)	(0.17)	(0.24)	(0.10)	(0.13)	(0.04)	(8)	(24)	(12)
p	<0.001	NS	NS	<0.001	<0.02	NS	NS	NS

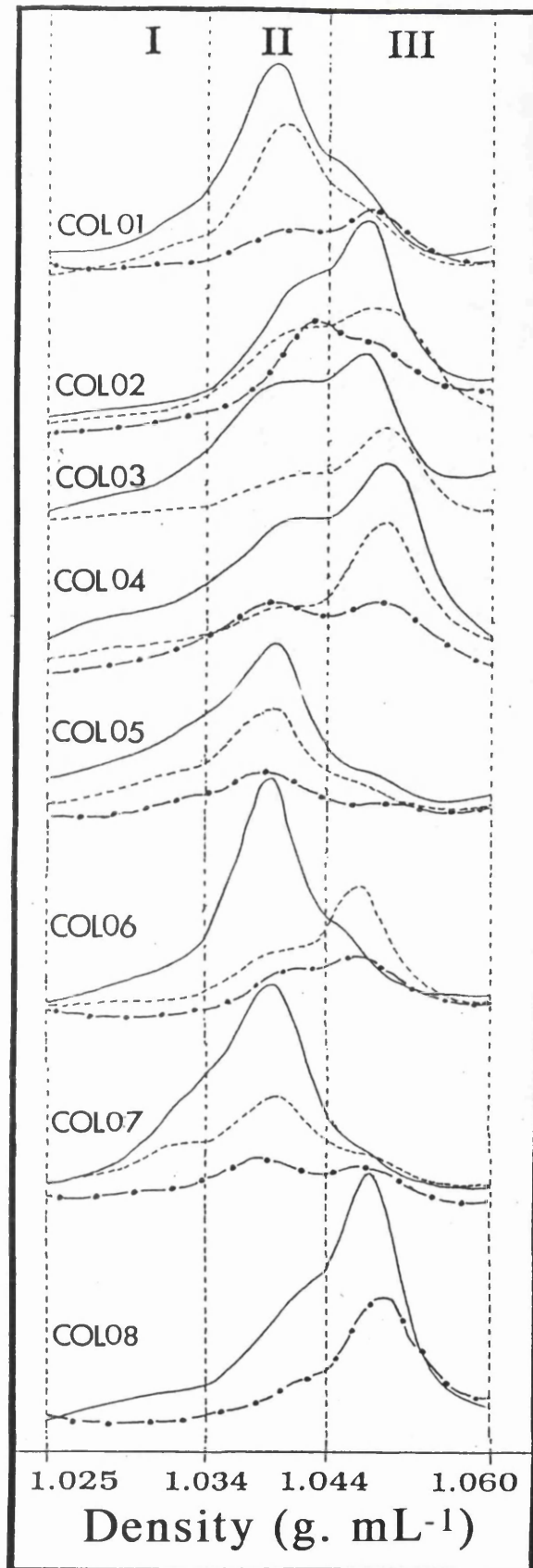
\* Cholesterol, triglyceride, VLDL, LDL and HDL-C figures are means of three measurements over 13 days.

**Table 26.** LDL Subfraction Changes with Colestipol and Simvastatin Therapy

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100mL plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
Mean	413	74	222	118
(SEM)	(32.6)	(8.5)	(16.4)	(29.5)
<i>On Colestipol and Simvastatin</i>				
COL 01C	156	14	75	67
COL 02C	154	26	77	51
COL 03C	168	12	69	88
COL 04C	232	24	80	128
COL 05C	195	44	121	30
COL 06C	195	17	93	85
COL 07C	190	27	111	52
COL 08C	227	0	73	154
Mean	190	21	87	82
(SEM)	(10.5)	(4.6)	(6.8)	(14.7)
p*	<0.001	<0.001	<0.001	NS

\* Comparison of the means was performed using paired students t-test.

by combined therapy, but HDL-cholesterol showed a significant increase (10%,  $p < 0.02$ ). Lp(a) levels were unchanged from baseline by combination therapy. Centrifugal analysis of the subfraction distribution in the LDL and HDL density intervals was also performed before and during therapy. HDL<sub>2</sub> and HDL<sub>3</sub> masses were unaffected by therapy as was the HDL<sub>2/3</sub> ratio. Figure 54 shows the LDL subfraction profile obtained upon density gradient fractionation of subjects' plasma. The pattern exhibited considerable variation from subject to subject but in most three distinct populations of particles could be distinguished both before and during therapy. There was a marked fall in total LDL mass (54%,  $p < 0.001$ ), which could be explained by profound decreases in both of the larger, more buoyant species LDL-I and LDL-II (72%,  $p < 0.001$  and 61%,  $p < 0.001$  respectively). Despite a 31% reduction in the mean value of the smaller, denser LDL-III (table 26), the overall effect was not statistically significant. Compositional analysis of the four major apoB containing lipoprotein classes are summarised in table 27.



**Figure 54.** LDL subfraction profiles at baseline (—), during colestipol therapy (---) and on combined colestipol and simvastatin therapy (---) in subjects COL 01-COL 08.

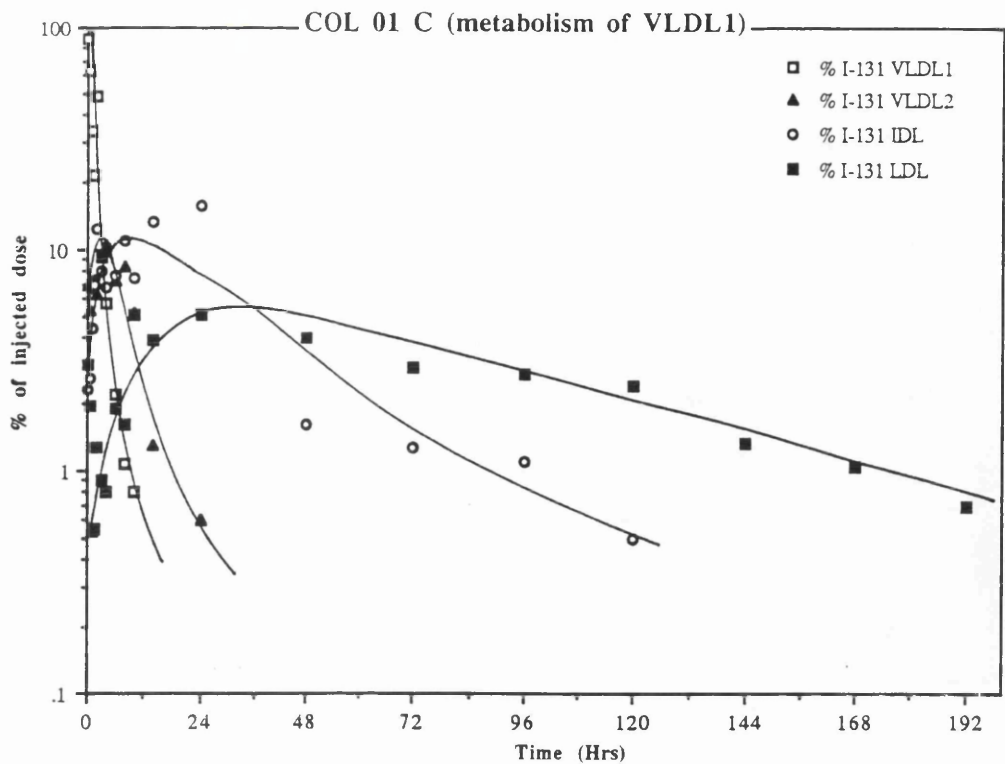


Combination therapy did not affect the absolute composition of either VLDL<sub>1</sub> or VLDL<sub>2</sub>. However, there were marked changes in the composition of IDL and LDL with significant falls in all measured parameters. The cholesterol content of LDL fell by 51% but the apoB component fell only 44%. This resulted in a fall in the LDL cholesterol/apoB ratio and indicates the relative cholesterol depletion of these particles in response to combined drug therapy.

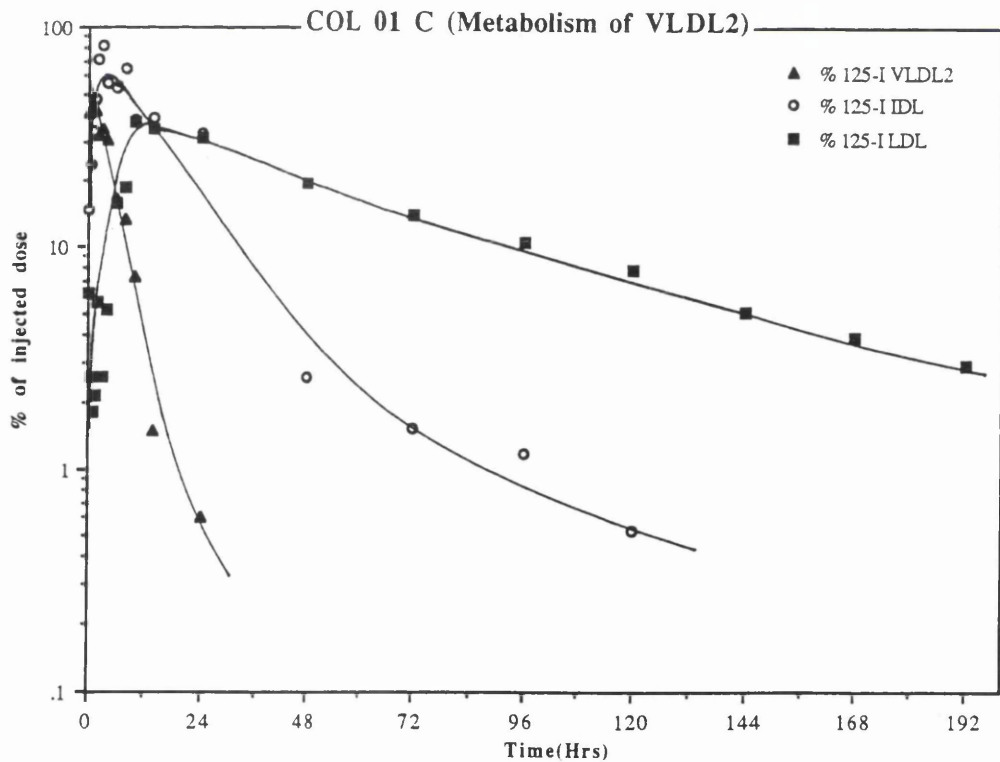
## 7.6 Apolipoprotein B Metabolism

The influence of colestipol and simvastatin therapy on apolipoprotein B metabolism was examined before and on the combined drug regimen (representative decay curves for one subject, COL 01 are shown in figures 33,34,55&56). Before therapy, apoB disappeared rapidly from the VLDL<sub>1</sub> flotation interval and appeared in VLDL<sub>2</sub> over a period of 6-18 h after injection (figure 33). The decay of radioactive apoB in VLDL<sub>2</sub> was slower than in VLDL<sub>1</sub>, taking almost 2 days to reduce to 1% of the injected dose (figure 34). Combination therapy with colestipol and simvastatin did not appear to increase the clearance rate of VLDL<sub>1</sub> apoB (figure 55) but in this individual the slope of the VLDL<sub>2</sub> apoB curve and therefore its clearance rate was increased (figure 56). The flux of apoB into IDL was comparable before and during therapy for both tracers. However, the decay rate of IDL apoB was much faster on combined drug therapy with the peak value attained in the LDL interval being consistently less than at baseline. Before therapy, LDL apoB radioactivity reached a maximum of 15% of injected dose about 48 h after VLDL<sub>1</sub> injection (figure 33), whereas during therapy (figure 55) the LDL apoB maximum was only 5%. These findings suggest that the metabolic fate of IDL apoB is markedly affected by therapy with more material being lost directly from the circulation rather than being converted to LDL. In this subject (COL 01) the clearance rate of LDL apoB radioactivity was also increased by combination therapy. Kinetic rate constants and apoB fluxes were derived by compartmental modelling. The results for all 8 subjects are summarised in table 28; decay curves for the other seven subjects and individual kinetic constants and masses are given are shown in appendix 3.

VLDL<sub>1</sub> apoB synthesis, pool size, and fractional transfer rate to VLDL<sub>2</sub> were unchanged from baseline by combination therapy. The VLDL<sub>2</sub> apoB pool size returned to pre-treatment levels with the addition of simvastatin to the regimen while the amount of material derived from VLDL<sub>2</sub> or by direct input was unaltered. IDL formation from VLDL<sub>2</sub>, presumably by delipidation was the same before and



**Figure 55.** On Colestipol and Simvastatin. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>



**Figure 56.** On Colestipol and Simvastatin. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject COL 01 after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

**Table 28a** VLDL<sub>1</sub> ApoB Metabolism before and on Colestipol & Simvastatin

	Synthesis (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To VLDL <sub>2</sub> (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
Mean (SEM)	914 (200)	112 (26)	2.53 (0.58)	6.08 (1.19)
<i>On Colestipol and Simvastatin</i>				
COL 01C	1150	131	7.64	1.14
COL 02C	543	66	1.54	6.69
COL 03C	697	89	1.34	6.50
COL 04C	598	74	1.95	6.01
COL 05C	506	47	2.38	8.40
COL 06C	720	108	1.03	5.64
COL 07C	419	34	1.10	11.22
COL 08C	1132	219	0.00	5.17
Mean (SEM)	720 (98)	96 (21)	2.12 (0.83)	6.35 (1.01)
p *	NS	NS	NS	NS
* Differences between the time points were assessed by the paired t-test.				

**Table 28b.** VLDL<sub>2</sub> ApoB Metabolism before and on Colestipol & Simvastatin

	Direct Synthesis (mg.d <sup>-1</sup> )	Flux from VLDL <sub>1</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux to IDL+ LDL (pools.d <sup>-1</sup> )
<i>Before Therapy</i>					
Mean (SEM)	576 (112)	534 (137)	308 (30)	0.94 (0.23)	3.07 (0.34)
<i>On Colestipol and Simvastatin</i>					
COL 01C	477	150	81	0.05	7.77
COL 02C	442	441	107	2.57	5.48
COL 03C	731	579	392	0.08	3.26
COL 04C	565	445	342	0.53	2.43
COL 05C	499	395	169	0.39	4.90
COL 06C	1207	609	326	0.26	5.34
COL 07C	1128	381	319	1.01	3.68
COL 08C	1309	1131	626	1.20	2.70
Mean (SEM)	795 (128)	516 (101)	295 (63)	0.76 (0.30)	4.45 (0.63)
p	NS	NS	NS	NS	NS

**Table 28c.** IDL ApoB Metabolism before and on Colestipol & Simvastatin.

	Flux from VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
Mean (SEM)	890 (96)	781 (58)	0.42 (0.08)	0.79 (0.06)
<i>On Colestipol and Simvastatin</i>				
COL 01C	624	349	0.98	0.81
COL 02C	596	281	0.51	1.61
COL 03C	1275	617	1.13	0.94
COL 04C	832	717	0.54	0.62
COL 05C	831	485	0.82	0.89
COL 06C	1733	541	1.88	1.32
COL 07C	1083	704	0.92	0.62
COL 08C	1691	342	3.80	1.15
Mean (SEM)	1083 (158)	505 (60)	1.32 (0.39)	1.00 (0.12)
p	NS	<0.005	<0.05	<0.05

**Table 28d.** LDL ApoB Metabolism before and on Colestipol & Simvastatin.

	Direct Synthesis ‡ (mg.d <sup>-1</sup> )	Flux from IDL + VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	VLDL-derived Plasma Pool (mg)	Fractional Catabolic Rate (pools.d <sup>-1</sup> )	Total ApoB Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>					
Mean (SEM)	300 (51)	651 (52)	2408 (279)	0.28 (0.01)	1790 (211)
<i>On Colestipol and Simvastatin</i>					
COL 01C	134	282	854	0.33	1761
COL 02C	70	461	1547	0.30	1055
COL 03C	62	565	1348	0.42	1490
COL 04C	182	442	1657	0.27	1336
COL 05C	218	432	1216	0.35	1223
COL 06C	112	717	1958	0.37	2039
COL 07C	75	525	1193	0.44	1622
COL 08C	8	391	2450	0.16	2449
Mean (SEM)	108 (24)	477 (46)	1528 (177)	0.33 (0.03)	1622 (161)
p	<0.01	<0.02	<0.002	NS	NS
‡ Direct synthesis in LDL was calculated as the difference between the total absolute catabolic rate (observed mass x overall FCR) and the input from VLDL and IDL.					



**Table 28e.** LDL ApoB Metabolism before and on Colestipol & Simvastatin.(continued)

	Total Plasma LDL pool (mg)	Total LDL Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>		
Mean (SEM)	3470 (367)	951 (79)
<i>On Colestipol and Simvastatin</i>		
COL 01C	1260	416
COL 02C	1779	531
COL 03C	1496	627
COL 04C	2330	624
COL 05C	1838	650
COL 06C	2260	829
COL 07C	1363	600
COL 08C	2498	399
Mean (SEM)	1853 (166)	585 (49)
p	<0.001	<0.003

during combination therapy. The IDL apoB plasma pool size fell significantly from 781 to 505mg (35%,  $p<0.005$ ) due to an increase in direct catabolism (214%,  $p<0.05$ ) and fractional transfer rate into LDL (27%,  $p<0.05$ ) of IDL apoB. The total LDL apoB plasma pool showed a highly significant fall, in response to colestipol and simvastatin therapy, from 3427 to 1853mg ( $p<0.001$ ). The calculated LDL apoB pool, i.e. that derived from VLDL was also reduced by therapy from 2408 to 1528mg ( $p<0.002$ ) due to a combination of reduced synthetic and increased catabolic rates. The fractional catabolic rate of LDL apoB, rose in 6 of the 8 patients but because of the scatter of responses this did not achieve overall significance. In this relatively small group of subjects it was the reduction in LDL apoB synthesis rather than the change in catabolism that appeared to be the predominant factor in reducing the plasma LDL-cholesterol level. Total LDL apoB synthesis fell by 39% ( $p<0.005$ ) and direct synthesis of LDL apoB fell by 64% ( $p<0.01$ ). It is also of note that the mean calculated total apoB synthetic rate was unchanged with combination therapy (table 28d). Figures 57 and 58 summarise the

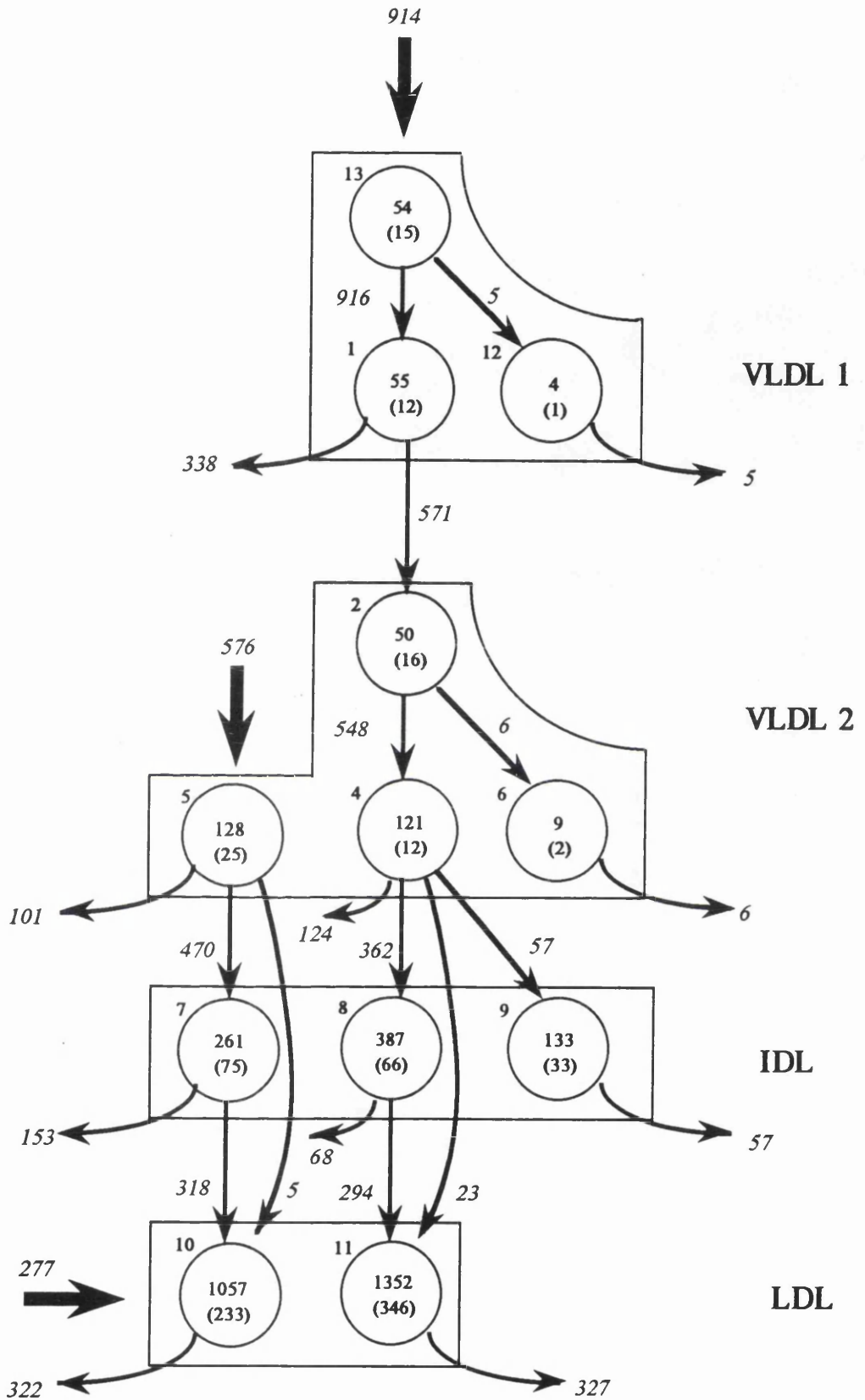
flux of apoB through the delipidation cascade at baseline and on combination therapy.

## 7.7 Discussion

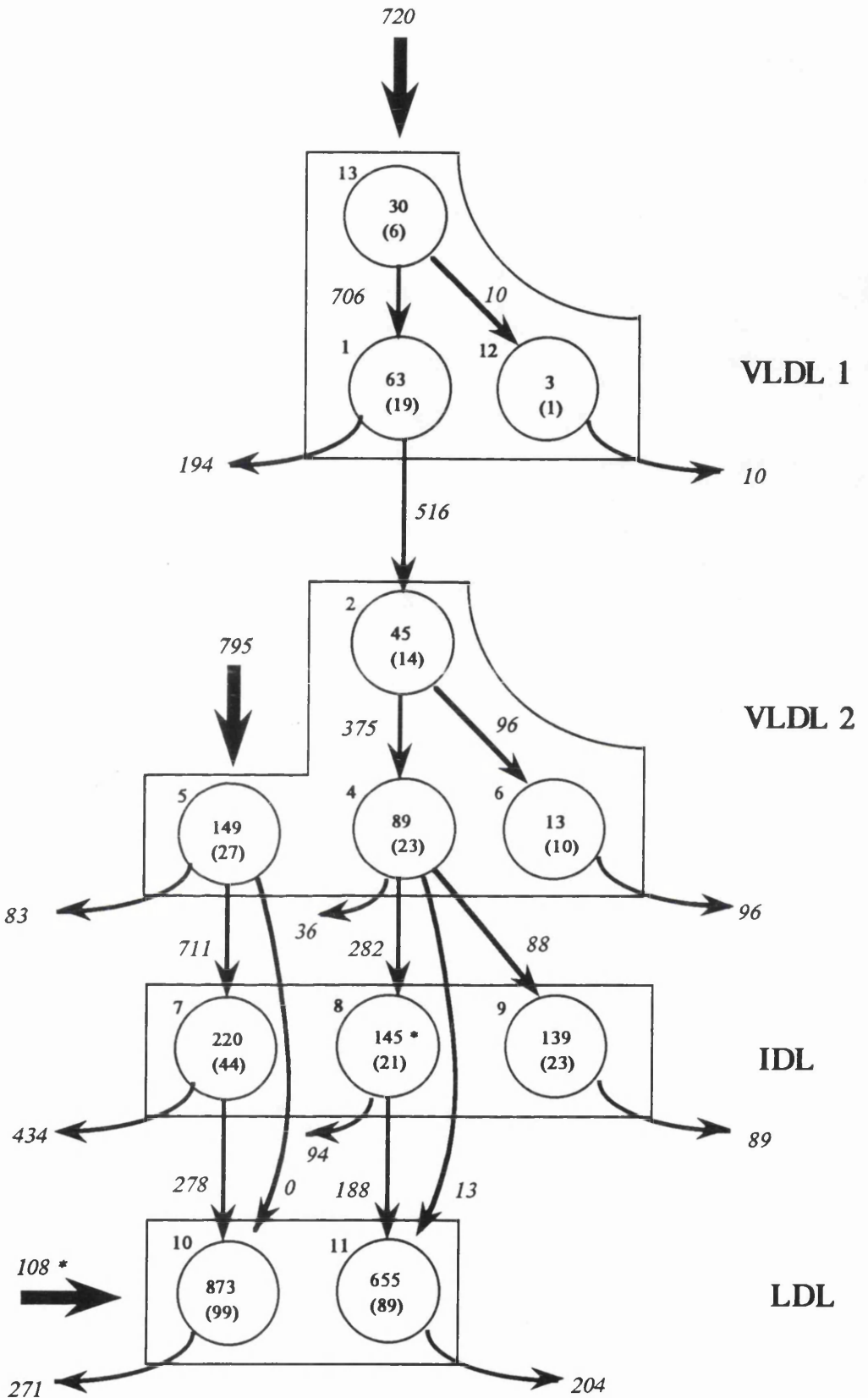
The lipid lowering efficacy of the combination of a bile acid sequestrant resin and an HMG CoA-reductase inhibitor has been clearly demonstrated here with results that are in line with previous studies. This combination has been examined with favourable results in a number of clinical studies in the US and Japan (Mabuchi *et al* 1983, Illingworth 1984, Leren *et al* 1988). The improvement in the lipid and lipoprotein profile reported here is consistent with the results of these and other studies, where LDL-cholesterol levels have fallen in the order of 50%. Similarly, significant increases in HDL-cholesterol, as shown here, have also been reported by Vega & Grundy (1987) and Leren *et al* (1988). It is interesting to note that the latter group also observed no change in Lp(a) level in response to combined lovastatin and cholestyramine therapy. Consistent Lp(a) findings are reported in the present study with the closely related combination of simvastatin and colestipol.

This combination is attractive to the clinician in that there is a good theoretical basis for its improved efficacy over monotherapy with either drug. When bile acid sequestrant resins are administered, the hepatic bile acid pool is depleted and the hepatocytes are thought to respond by up-regulating their cell surface apoB/E receptors. This promotion of receptor-mediated LDL clearance is somewhat off-set by a concomitant increase in endogenous cholesterol biosynthesis. By co-administration of an HMG CoA-reductase inhibitor this effect is minimised and a greater level of apoB/E receptor activity achieved. The clinical result is a profound lowering of LDL-cholesterol by as much as 50-70%.

If this combination does cause a increase in apoB/E receptor expression it would be expected to result in an increase in LDL apoB clearance. This was clearly observed in FH heterozygotes (Bilheimer *et al* 1983, Grundy *et al* 1985, Vega *et al* 1989), but even in this class of subject a reduction in apo-LDL synthesis has been required to explain the kinetic findings (Grundy *et al* 1985). When patients without the specific defect of lipoprotein catabolism seen in FH are examined, the principal effect of this combined regimen is a fall in apo-LDL production (Vega & Grundy 1987) although an increase in apo-LDL FCR, and compositional changes in the LDL particle are also instrumental in causing an overall reduction in the plasma LDL-cholesterol level. The marked increase in IDL apoB catabolism observed in



**Figure 57.** Summary of apoB metabolism at baseline. Numbers in circles represent apoB pool size [mean (SEM)] in mg. Numbers on arrows represent mean transfer of apoB in mg.d<sup>-1</sup>.



**Figure 58.** Summary of the effects of colestipol and simvastatin therapy on apoB metabolism. Numbers in circles represent apoB pool size [mean (SEM)] in mg. Numbers on arrows represent mean transfer of apoB in mg·d<sup>-1</sup>. \* significantly different from control  $p < 0.05$ .

the present study is entirely consistent with enhanced apoB/E receptor activity, for the IDL particle, with its greater apoE content, is considered to be a more appropriate ligand for this receptor than LDL itself. As a result of this increased removal of IDL apoB there was a fall in the throughput of material into the LDL density interval. This fall in VLDL-derived LDL synthesis was complemented by a drug induced fall in LDL direct synthesis. These two changes, together resulted in a highly significant fall in total LDL apoB synthesis ( $p < 0.003$ ). In 6 of the 8 subjects studied there was also an increase in LDL apoB FCR. This is entirely consistent with the findings of Vega & Grundy (1987) who observed an increase in the apo-LDL FCR in 8 of the 10 subjects with primary, moderate hypercholesterolaemia they studied. These workers also reported compositional changes in LDL, which they attributed to combination drug therapy and used to explain in part the fall in plasma LDL-cholesterol they observed. In the present study there was a fall in cholesterol/protein ratio in both IDL and LDL indicating that while the number of particles fell there was also some degree of cholesterol depletion in these species. This is consistent with the changes observed in these patients' LDL subfraction profiles, where there were significant falls in the larger, more cholesteryl ester rich species, LDL-I and LDL-II. Again, this is good evidence for the up-regulation of apoB/E receptors as the main mechanism of action of this combination regimen. Those LDL species that are relatively rich in cholesteryl ester are thought to bind to the apoB/E receptor with greater affinity (Koo *et al* 1988) and may be selectively removed from the circulation leaving smaller, denser LDL-III in relative abundance.

In conclusion, the combination of colestipol and simvastatin in this group of patients with primary moderate hypercholesterolaemia has been highly effective in correcting their aberrant lipid profile. The marked changes observed in LDL-cholesterol may be attributed to reduced LDL synthesis both from delipidation of VLDL and from *de novo* synthesis. The changes observed in the kinetics of IDL and LDL apoB, and the changes observed in lipoprotein composition and LDL subfraction profile are adequately explained by these drugs' proposed mechanism of action in enhancing apoB/E receptor function.

## *Chapter 8   Combination II Acipimox & Cholestyramine*

Experiment alone crowns the efforts of medicine, experiment limited only by the natural range of the powers of the human mind. Observation discloses in the animal organism numerous phenomena existing side by side, and interconnected now profoundly, now indirectly, or accidentally. Confronted with a multitude of different assumptions the mind must *guess* the real nature of this connection.

Ivan Pavlov

### 8.1 Introduction

Combination therapy with nicotinic acid or its derivatives and a bile acid sequestrant resin is one of the most effective lipid regulating therapies available and one of the first tried (Levy, Fredrickson & Schulman 1972). This regimen can reduce total and LDL-cholesterol by between 30-40% and is particularly attractive as both drugs have individually been shown to reduce coronary morbidity in large scale clinical trials (Coronary Drug Project 1975, Lipid Research Clinics 1984a & 1984b). This combined drug regimen has been shown to be effective in heterozygous familial hypercholesterolaemia (Packard *et al* 1980, Kane *et al* 1981) where normalization of LDL-cholesterol and regression of tendinous xanthomata have been reported. Regression of coronary atherosclerotic lesions has also been demonstrated using this combination of lipid lowering drugs. Blankenhorn *et al* (1987) reported in the Cholesterol Lowering Atherosclerosis Study (CLAS) a significant increase in regression and decrease in progression in subjects receiving niacin and colestipol therapy compared to placebo treated controls. These findings were confirmed by Brown *et al* (1990) in the Familial Atherosclerosis Treatment Study (FATS). Here

a combined regimen of niacin and colestipol was shown to be superior to lovastatin and colestipol in inducing coronary atherosclerosis regression.

Despite the clinical efficacy of combined nicotinic acid and sequestrant resin, many individuals are, however, unable to tolerate the side effects of nicotinic acid, which has prompted its substitution with the analogue, acipimox. This combination is reasonably well tolerated and is equally effective in reducing LDL-cholesterol levels (Series *et al* 1990).

The aim of the present study was to examine in detail the mechanism of action of combined bile acid sequestrant resin and nicotinic acid derivative therapy in patients with primary moderate hypercholesterolaemia. The results confirmed the significant lipid lowering that may be achieved using combination therapy and the kinetic analysis of apoB containing lipoproteins yielded interesting new findings.

## 8.2. Protocol

This extension of the study described in chapter 5 was designed to examine the influence of acipimox and cholestyramine on the metabolism of apoB containing particles. The preliminary screening period and baseline study period were as described in chapter 5. On completion of the second VLDL turnover study, the patients were commenced on cholestyramine 12g/day in addition to their acipimox therapy, 1250mg/day. The relatively low dose of cholestyramine was chosen to facilitate patient compliance. Patients remained on these doses for 10 weeks. During the final 2 weeks each patient underwent a third and final VLDL turnover investigation while on combined therapy. In addition lipid, lipoprotein and lipoprotein subfraction analyses were repeated at this time.

## 8.3 Subjects

The seven study patients were those described in chapter 5 and their characteristics are summarised in table 13. As before all concomitant medications were continued unchanged throughout this second study phase.

## 8.4 Adverse Events

Combination therapy with acipimox and cholestyramine was reasonably well tolerated by all subjects participating in the study. Three patients, APX 05, 06 and

07 complained of mild constipation after the addition of cholestyramine to their regimen, but this resolved after approximately the first two weeks of therapy and did not require any further intervention or interruption of therapy. Biochemical and haematological monitoring revealed no clinically significant changes from baseline attributable to therapy.

### 8.5 Lipids and Lipoproteins

Baseline plasma lipid and lipoprotein levels of those patients studied are shown in chapter 5, and their levels after combination therapy are displayed in table 29. Combined treatment reduced plasma cholesterol by 23% ( $p < 0.001$ ). This decrement was due in this group to a 27% fall in LDL-cholesterol ( $p < 0.003$ ), and a 19% fall in VLDL-cholesterol ( $p < 0.02$ ). Triglyceride and HDL-cholesterol were not significantly affected by combined therapy, nor was the plasma level of Lp(a). Centrifugal analysis of the subfraction distribution in the LDL and HDL density intervals was performed before and during therapy but neither HDL<sub>2</sub> and HDL<sub>3</sub> masses nor the HDL<sub>2/3</sub> ratio were affected by therapy. Figure 59 shows the LDL subfraction profile obtained upon density gradient fractionation of each subject's plasma. The pattern exhibited considerable variation from subject to subject but in most three distinct populations of particles could be distinguished both before and during therapy. There was some shift in the LDL subfraction pattern towards a larger more buoyant species with a rise in LDL-I of 33% and a fall in LDL-III of 49% (table 30). However, the scatter of responses in this small group was such that these changes did not reach statistical significance. Compositional analysis of the four major apoB containing lipoprotein classes are summarised in table 31. Combination therapy did not affect the absolute composition of either VLDL<sub>1</sub> or VLDL<sub>2</sub>. However, there were marked changes in the chemical analysis of IDL and LDL with significant falls in the absolute value of the measured lipids and protein in LDL. When the relative lipid composition of these lipoproteins were considered it appeared that while there was a significant reduction in the number of circulating particles, as evidenced by the fall in IDL and LDL apoB, the make up of these particles was largely unchanged with the exception of cholesteryl ester content. In IDL this rose from 38 to 44% and in LDL the rise was from 36 to 40%.

### 8.6 Apolipoprotein B Metabolism

The influence of acipimox and cholestyramine therapy on apolipoprotein B metabolism was examined before and on the combined drug regimen



Table 29. Lipid and Lipoprotein Changes with Combined Acipimox and Cholestyramine Therapy

Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	HDL <sub>2</sub> (mass)	HDL <sub>3</sub> (mass)	Lp(a)
			-----mmol. L <sup>-1</sup> -----			-----mg. dL <sup>-1</sup> -----		mg. dL <sup>-1</sup>
<i>Before Therapy</i>								
Mean	7.9	2.02	0.97	5.75	1.15	51	228	23
(SEM)	(0.2)	(0.21)	(0.12)	(0.20)	(0.09)	(11)	(20)	(11)
<i>On Acipimox and Cholestyramine</i>								
APX 01C	6.0	2.43	1.03	4.85	1.08	23	236	5
APX 02C	5.6	1.00	0.48	3.68	1.48	73	254	3
APX 03C	5.5	1.13	0.48	3.42	1.60	101	242	65
APX 04C	6.1	2.62	1.03	3.97	1.07	29	238	2
APX 05C	6.0	1.90	0.65	4.15	1.22	33	272	73
APX 06C	6.6	2.53	1.08	4.48	1.00	37	206	28
APX 07C	7.0	1.42	0.78	4.75	1.43	98	260	1
Mean	6.1	1.86	0.79	4.19	1.27	56	244	25
(SEM)	(0.2)	(0.26)	(0.10)	(0.20)	(0.09)	(13)	(8)	(12)
p	<0.001	NS	<0.02	<0.003	NS	NS	NS	NS

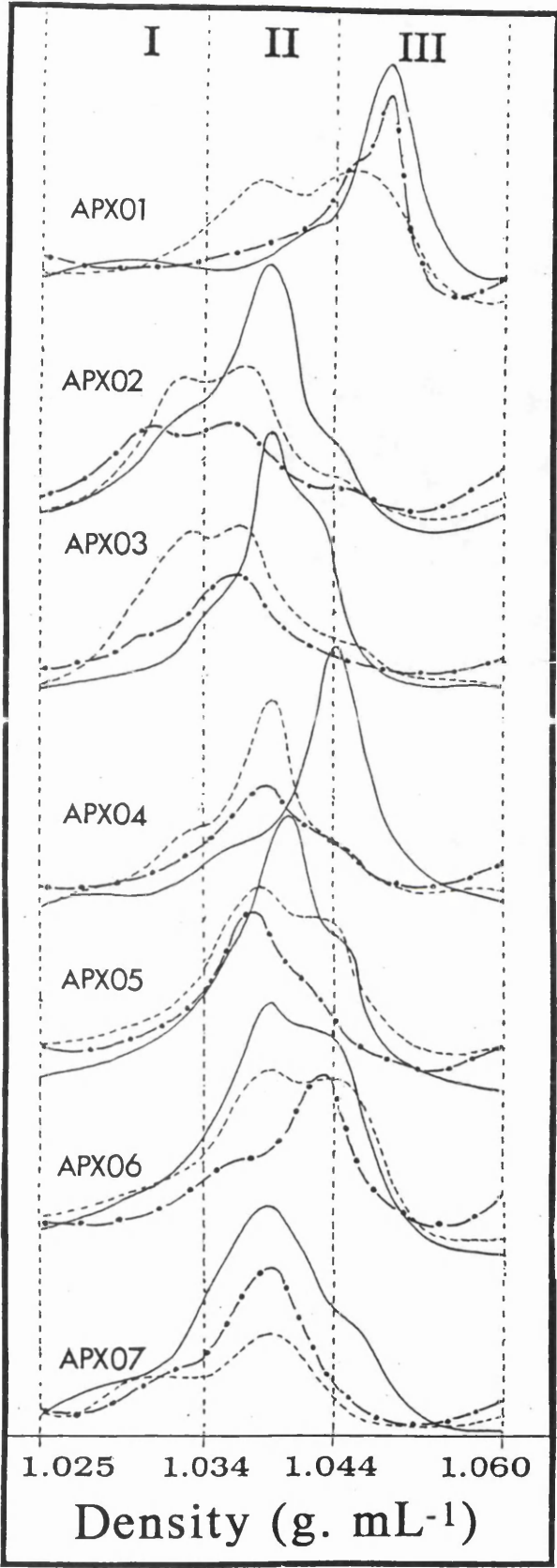
\* Cholesterol, triglyceride, VLDL, LDL and HDL-C figures are means of three measurements over 13 days.

**Table 30.** LDL Subfraction Changes with Acipimox and Cholestyramine Therapy

Subject	Tot LDL	LDL-I	LDL-II	LDL-III
	-----mg lipoprotein. 100mL plasma <sup>-1</sup> -----			
<i>Before Therapy</i>				
Mean (SEM)	416 (40.3)	51 (13.8)	214 (42.2)	151 (56.8)
<i>On Acipimox and Cholestyramine</i>				
APX 01C	378	83	136	159
APX 02C	268	59	147	62
APX 03C	261	60	157	44
APX 04C	274	115	134	25
APX 05C	296	18	124	154
APX 06C	305	40	219	46
APX 07C	368	103	239	26
Mean (SEM)	307 (18)	68 (13)	165 (17)	77 (22)
p*	NS	NS	NS	NS

\* Comparison of the means was performed using paired students t-test.

(representative decay curves for one subject, APX 03 are shown in figures 39,40, 60 & 61). Before therapy apoB disappeared rapidly from the VLDL<sub>1</sub> flotation interval and appeared in VLDL<sub>2</sub> over a period of 4-6 h after injection (figure 39). The decay of radioactive apoB in VLDL<sub>2</sub> was much slower than in VLDL<sub>1</sub>, taking approximately 60 hours to reduce to 1% of the injected dose (figure 40). Combination therapy with acipimox and cholestyramine did not appear to increase the clearance rate of VLDL<sub>1</sub> apoB (figure 60) but in this individual the slope of the VLDL<sub>2</sub> apoB curve and therefore its clearance rate was increased (figure 61). The flux of apoB into IDL was affected by therapy for both tracers but in different ways. VLDL<sub>1</sub> derived apoB appeared to enter the IDL fraction more quickly on therapy and achieve a higher peak value (60% versus 40% of injected dose) (figure 60). The decay rate of this VLDL<sub>1</sub> derived apoB was also initially slower than at baseline. VLDL<sub>2</sub> derived apoB achieved a smaller peak value in the IDL fraction on combined therapy when compared to the control phase, but its rate of decay was indistinguishable (figure 61). In this subject (APX 03) the clearance rate of LDL

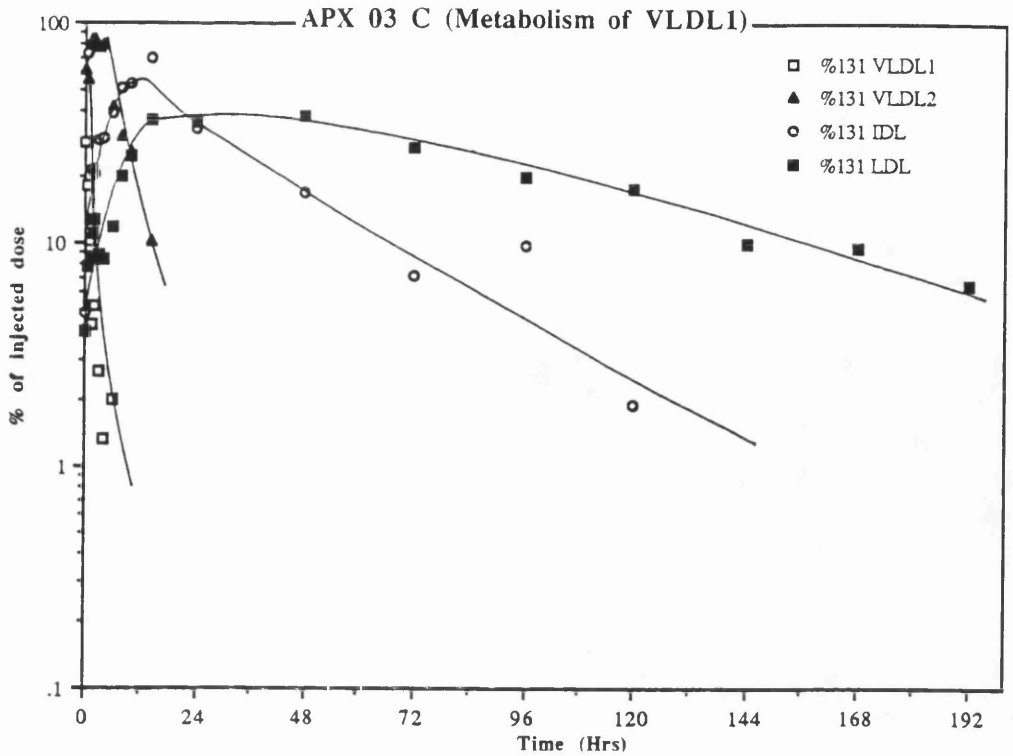


**Figure 59.** LDL subfraction profiles at baseline (—), during acipimox therapy (---) and on combined acipimox and cholestyramine therapy (-.-) in subjects APX 01-APX 07.

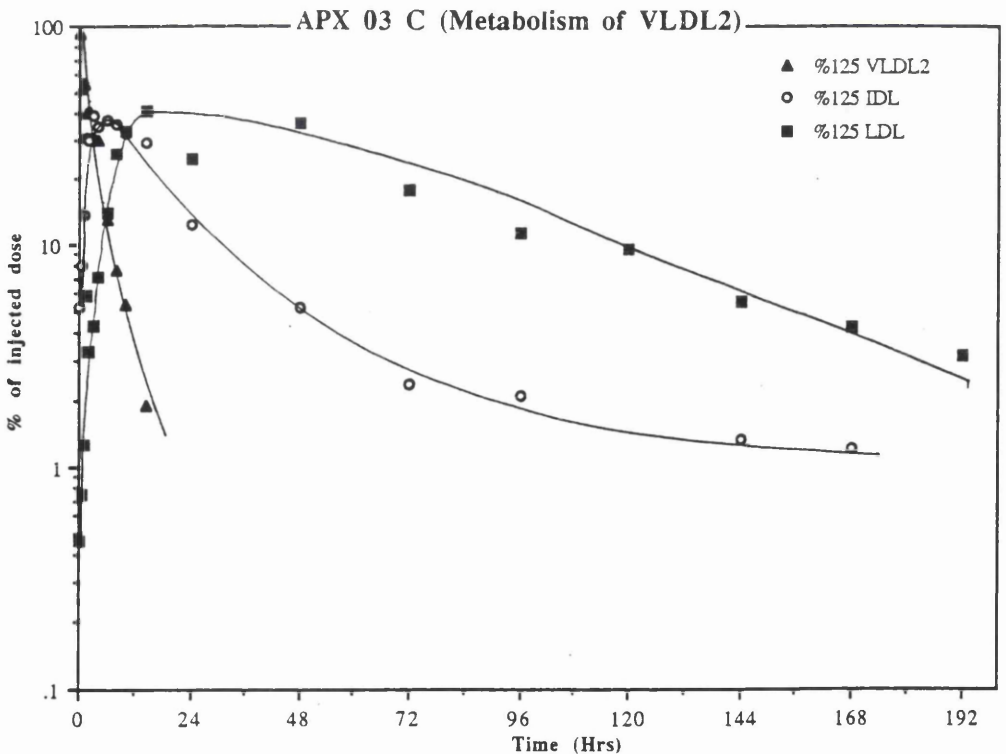
**Table 31. Composition of Apolipoprotein B Containing Lipoproteins Before and On Acipimox and Cholestyramine Therapy.**

	Free Cholesterol	Cholesteryl Ester	Triglyceride (mg. dL <sup>-1</sup> )	Phospholipids	Apo B
VLDL <sub>1</sub>	Before Therapy On Therapy	5.33 (2.09)* 3.95 (1.18)	81.20 (15.86) 7.94 (14.35)	23.79 (4.69) 18.56 (3.63)	4.95 (0.74) 3.56 (0.93)
VLDL <sub>2</sub>	Before Therapy On Therapy	6.05 (0.66) 3.77 (0.60)†	32.05 (2.46) 21.81 (3.06)†	22.36 (3.47) 12.61 (2.01)	7.40 (0.45) 2.96 (0.54)†
IDL	Before Therapy On Therapy	8.09 (0.81) 5.50 (0.77)§	11.80 (1.06) 8.28 (0.82)	25.39 (4.54) 16.25 (1.23)	10.43 (0.92) 7.38 (1.27)§
LDL	Before Therapy On Therapy	43.10 (5.07) 28.67 (3.74)§	21.62 (1.80) 14.10 (0.89)†	86.21 (6.82) 64.00 (4.58)§	104.34 (11.03) 70.39 (5.64)§

\* Mean (SEM)  
† Significantly different from baseline, †p<0.005, ‡p<0.01, §p<0.05 by paired student's t-test



**Figure 60.** On Acipimox and Cholestyramine. Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [ $^{131}\text{I}$ ]-VLDL<sub>1</sub>



**Figure 61.** On Acipimox and Cholestyramine Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL, and LDL in subject APX 03 after injection of autologous [ $^{125}\text{I}$ ]-VLDL<sub>2</sub>.

apoB radioactivity was also unaffected by combination therapy. Kinetic rate constants and apoB fluxes were derived by compartmental modelling. The results for all 7 subjects are summarised in table 32; decay curves for the other six subjects and individual kinetic constants and masses are given are shown in appendix 4.

VLDL<sub>1</sub> apoB synthesis, pool size, and fractional transfer rate to VLDL<sub>2</sub> were unchanged from baseline by combination therapy. Despite the latter, the VLDL<sub>2</sub> apoB pool size fell by 36% ( $p<0.03$ ), due primarily to a significant fall in the absolute apoB mass derived from VLDL<sub>1</sub> (34%,  $p<0.03$ ). Direct input of VLDL<sub>2</sub> apoB was unaltered, but the fractional transfer rate from VLDL<sub>2</sub> into IDL was markedly increased from 2.12 to 3.54 pools.  $d^{-1}$  (67%,  $p<0.005$ ), although the absolute mass of apoB transferred into the IDL density interval was unaffected by the combined regimen. The IDL apoB plasma pool and FCR were unchanged by therapy but the fractional transfer of IDL apoB into LDL was significantly increased (26%,  $p<0.05$ ). The total LDL apoB plasma pool showed a significant fall, in response to acipimox and cholestyramine therapy, from 3480 to 2728mg ( $p<0.02$ ), but the calculated LDL apoB pool, i.e. that derived from VLDL was relatively unchanged. The fractional catabolic rate of LDL apoB, rose in 5 of the 7 patients but because of the scatter of responses this did not achieve overall significance.. Total LDL apoB synthesis was unaffected by therapy as was the direct synthesis of LDL apoB. In this relatively small group of subjects, it appeared to be the collective result of a number of small and statistically insignificant changes in both synthetic and catabolic parameters that contributed to the observed fall in plasma LDL-cholesterol. Once again, as in all previous chapters, it is interesting to note that the mean calculated total apoB synthetic rate remained constant with combination therapy (table 32). Figures 43 and 62 summarise the flux of apoB through the delipidation cascade at baseline and on combination therapy.

## 8.7 Discussion

The 27% fall in mean LDL-cholesterol observed in this group of patients is highly significant both statistically and clinically. It is in accord with the findings of Series *et al* (1990) who reported the effects of the same combination of acipimox and cholestyramine in a larger group of patients with type II hyperlipoproteinaemia. In the present study no significant change was noted in HDL-cholesterol or in the masses of the HDL subfractions as have been described for both acipimox and resin (Series *et al* 1990) and nicotinic acid and resin (Packard *et al* 1980). There was however, a 10.4 % rise in HDL-cholesterol in response to combination therapy and

**Table 32a** VLDL<sub>1</sub> ApoB Metabolism before and on Acipimox & Cholestyramine

	Synthesis (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To VLDL <sub>2</sub> (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
Mean (SEM)	762 (154)	139 (31)	3.41 (1.35)	3.65 (0.87)
<i>On Acipimox and Cholestyramine</i>				
APX 01C	1098	238	2.26	2.35
APX 02C	1404	58	21.52	2.69
APX 03C	409	31	3.46	9.74
APX 04C	330	69	2.65	2.13
APX 05C	439	77	1.39	4.32
APX 06C	222	68	1.00	2.26
APX 07C	956	65	10.72	3.99
Mean (SEM)	694 (172)	87 (26)	6.14 (2.85)	3.93 (1.02)
p *	NS	NS	NS	NS
* Differences between the time points were assessed by the paired student's t-test.				

**Table 32b.** VLDL<sub>2</sub> ApoB Metabolism before and on Acipimox & Cholestyramine.

	Direct Synthesis (mg.d <sup>-1</sup> )	Flux from VLDL <sub>1</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux to IDL+ LDL (pools.d <sup>-1</sup> )
<i>Before Therapy</i>					
Mean (SEM)	696 (110)	414 (68)	414 (63)	0.62 (0.17)	2.12 (0.25)
<i>On Acipimox and Cholestyramine</i>					
APX 01C	624	560	477	0.57	1.92
APX 02C	653	156	246	0.53	2.77
APX 03C	1941	302	329	2.26	4.56
APX 04C	452	147	181	0.24	3.07
APX 05C	938	333	219	0.58	5.22
APX 06C	584	154	188	0.34	3.57
APX 07C	792	259	217	1.14	3.69
Mean (SEM)	855 (190)	273 (56)	265 (40)	0.81 (0.27)	3.54 (0.42)
p	NS	<0.03	<0.03	NS	<0.005

**Table 32c.** IDL ApoB Metabolism before and on Acipimox & Cholestyramine.

	Flux from VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	Plasma Pool (mg)	Direct Catabolism (pools. d <sup>-1</sup> )	Flux To LDL (pools. d <sup>-1</sup> )
<i>Before Therapy</i>				
Mean (SEM)	813 (100)	795 (73)	0.28 (0.03)	0.75 (0.08)
<i>On Acipimox and Cholestyramine</i>				
APX 01B	913	880	0.26	0.78
APX 02B	672	751	0.38	0.51
APX 03B	1460	776	0.57	1.31
APX 04B	529	443	0.23	0.97
APX 05B	1141	460	0.97	1.51
APX 06B	671	896	0.15	0.59
APX 07B	794	743	0.16	0.91
Mean (SEM)	883 (122)	707 (70)	0.39 (0.11)	0.94 (0.14)
p	NS	NS	NS	<0.04

**Table 32d.** LDL ApoB Metabolism before and on Acipimox & Cholestyramine.

	Direct Synthesis ‡ (mg.d <sup>-1</sup> )	Flux from IDL + VLDL <sub>2</sub> (mg.d <sup>-1</sup> )	VLDL derived Plasma Pool (mg)	Fractional Catabolic Rate (pools.d <sup>-1</sup> )	Total ApoB Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>					
Mean (SEM)	321 (95)	598 (89)	2332 (276)	0.26 (0.03)	1778 (321)
<i>On Acipimox and Cholestyramine</i>					
APX 01C	232	685	2526	0.27	1954
APX 02C	276	395	1261	0.31	2333
APX 03C	507	1056	2692	0.39	2857
APX 04C	161	454	1595	0.28	943
APX 05C	33	696	2496	0.28	1410
APX 06C	0	532	1809	0.29	806
APX 07C	154	681	2458	0.28	1902
Mean (SEM)	195 (64)	643 (83)	2120 (210)	0.30 (0.02)	1744 (280)
p	NS	NS	NS	NS	NS
‡ Direct synthesis in LDL was calculated as the difference between the total absolute catabolic rate (observed mass x overall FCR) and the input from VLDL and IDL.					

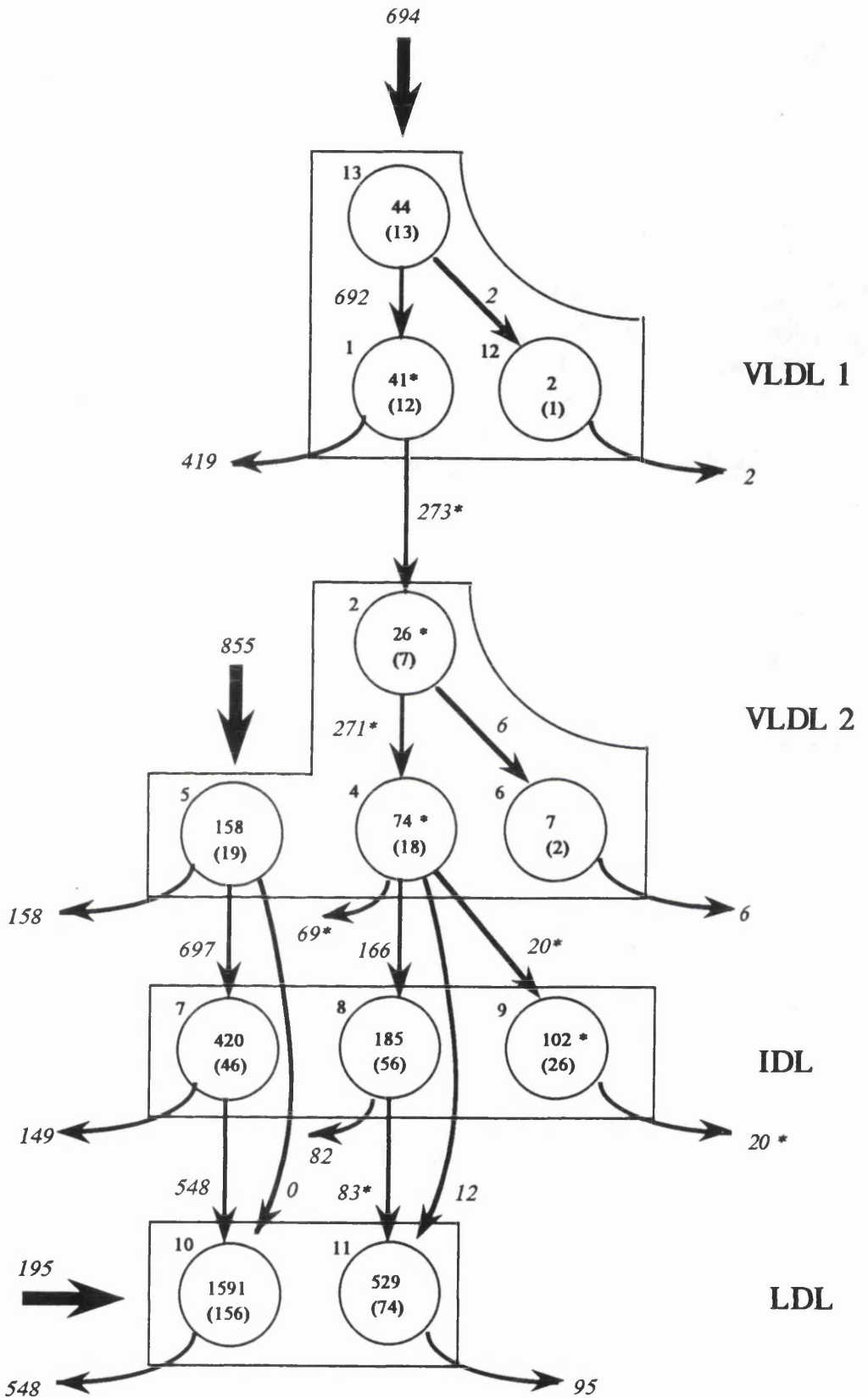


**Table 32e.** LDL ApoB Metabolism before and on Acipimox & Cholestyramine (cont)

	Total Plasma LDL pool (mg)	Total LDL Synthesis (mg.d <sup>-1</sup> )
<i>Before Therapy</i>		
Mean (SEM)	3480 (359)	919 (149)
<i>On Acipimox and Cholestyramine</i>		
APX 01C	3384	917
APX 02C	2152	671
APX 03C	3991	1563
APX 04C	2170	615
APX 05C	2614	729
APX 06C	1777	532
APX 07C	3008	835
Mean (SEM)	2728 (295)	837 (130)
p	<0.02	NS

this just fell short of statistical significance ( $p=0.09$ ). This lack of effect on HDL metabolism is readily explained when the relatively small sample size studied and their inherently diverse underlying lipoprotein metabolism are considered.

The use of this combination of lipid lowering drugs has a sound theoretical basis. Bile acid sequestrant resin therapy, as described in chapter 4, exerts its lipid lowering effects by interrupting the enterohepatic circulation of bile acids. This results in depletion of the regulatory sterol pool in the hepatocyte and increased expression of apoB/E receptors. A homeostatic side effect of resin therapy is increased hepatic cholesterologenesis, secondary to the activation of the liver enzyme HMG CoA reductase. Nicotinic acid and its analogue, acipimox, exert their hypolipidaemic effect by inhibiting peripheral lipolysis and thereby reducing free fatty acid flux to the liver where VLDL triglyceride synthesis is curtailed. This action is thought not only to minimise the resin-induced hypertriglyceridaemia, but



**Figure 62.** Summary of the effects of acipimox and cholestyramine therapy on apoB metabolism. Numbers in circles represent apoB pool size [mean (SEM)] in mg. Numbers on arrows represent mean transfer of apoB in mg.d<sup>-1</sup>. \* significantly different from control  $p < 0.05$ .

also to enhance the cholesterol lowering effect of the resins by reducing the input into the VLDL-IDL-LDL delipidation cascade.

The relative compositional changes noted here are interesting because they would not support enhanced apoB/E receptor activity as a means of explaining the lipid lowering seen with this regimen. The apoB/E receptor is thought to selectively remove cholesteryl ester rich particles from the circulation (chapter 3). While colestipol on its own would be expected to enhance apoB/E receptor activity, an increase in these cholesteryl ester rich species, as seen here and as opposed to a fall in their number, would seem to counter this explanation.

The effects of acipimox and cholestyramine in combination on the LDL subfraction profile are not surprising when the individual effects of these drugs are considered. Acipimox, in common with other triglyceride-lowering drugs shifts the LDL subfraction profile towards the lighter and larger species (chapter 5), while the bile acid sequestrant resin, colestipol, has been shown, in chapter 4, to have the opposite effect. When cholestyramine was added to the regimen it appeared to completely offset the dramatic shift in LDL subfractions seen with acipimox alone (chapter 5), although there was a further fall in total LDL mass. Acipimox therapy appeared to favour the formation of potentially apoB/E receptor active LDL species viz. LDL-I & LDL-II. It may be speculated that as cholestyramine enhances apoB/E receptor expression these species will be quickly removed from the circulation resulting in an apparent reversal of the subfraction profile. Such an hypothesis may explain the apparent clinical efficacy of a similar combined regimen (colestipol & niacin) as used in the 'CLAS' study (Blankenhorn *et al* 1987).

As described in chapter 5 there is virtually no literature describing the influence of acipimox on lipoprotein kinetics, and there is none at all reporting the effects of combined acipimox and bile acid sequestrant resin therapy. When a drug that is thought to enhance clearance of apoB containing lipoproteins yet stimulate cholesterol synthesis is given in conjunction with a drug that has no known effect on apoB/E receptor function but has the disparate effects of reducing VLDL production and inhibiting lipolysis at the lower end of the delipidation cascade, it may be expected that there will be some confusion in trying to unravel their composite metabolic effects. This has indeed been the case. There appeared to be remnants of the action of acipimox on the delipidation cascade i.e. reduced total VLDL pool size and enhanced fractional transfer of apoB from VLDL<sub>2</sub> to IDL, and there were findings that demonstrated the additive effects of the drugs e.g. the flux

of apoB from VLDL<sub>1</sub> to VLDL<sub>2</sub>, which fell on acipimox therapy and fell further on combination therapy to achieve a statistically significant fall from baseline. There were also anomalous findings e.g. the slight increases in total LDL synthesis and observed LDL apoB mass seen when cholestyramine was added to the regimen. Such apparently discordant findings are difficult to explain in the context of the present study and will only be resolved by larger studies, or a similar study in a group with less heterogeneous hyperlipidaemia.

In conclusion, combination therapy with acipimox and cholestyramine therapy in primary moderate hypercholesterolaemia has been shown to be highly effective in lowering total and LDL-cholesterol. In contrast to other studies no significant change was observed in HDL-cholesterol or in HDL subfraction mass. This lack of response may be explained by the small group size studied. The investigation of apoB metabolism in these patients provided equivocal yet interesting findings. A combination of reduced synthesis and increased catabolism of apoB containing LDL precursors and of LDL itself has to be invoked to explain the observed fall in plasma LDL-cholesterol.

## Chapter 9 Cholesterol Biosynthesis

I must begin with a good body of facts and not from a principle (in which I always suspect some fallacy) and then as much deduction as you please.

Charles Darwin

### 9.1 Introduction

Because the classical method of assessing whole body cholesterol synthesis in humans, involving sterol balance studies, is laborious and insensitive to acute changes in cholesterol synthesis, alternative methods have been sought. Serum or plasma levels of various cholesterol precursors have been proposed as indirect indices of cholesterol biosynthesis viz. free methylsterols (Miettinen 1970, 1982), squalene (Nestel & Kudchodkar 1975), mevalonic acid (Parker *et al* 1982) and most recently total lathosterol (Bjorkhem *et al* 1987, Kempen *et al* 1988). Only the latter two, mevalonic acid and lathosterol have stood the test of time. However, they are very different compounds from opposite ends of the cholesterol biosynthetic pathway determined by very different assay systems. To investigate the effects of the lipid lowering drugs, simvastatin and fenofibrate, assays for these cholesterol precursors were established. Because these assays are infrequently used they are described in detail in chapter 2.

Mevalonic acid, an intermediate of cholesterol biosynthesis, is the immediate product of the important rate-limiting step catalysed by HMG CoA reductase (Popjak & Cornforth 1960), as shown in figure 63.

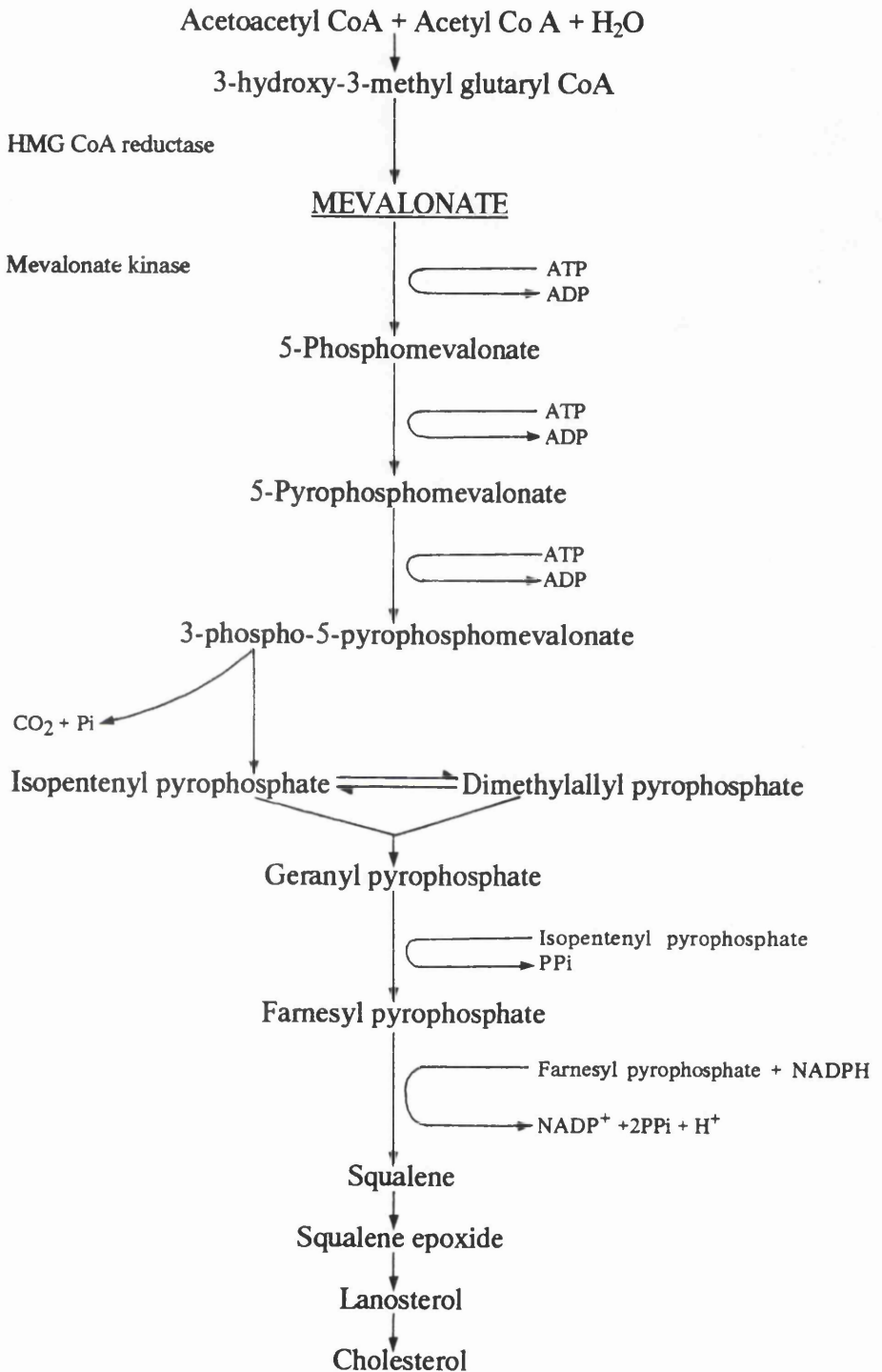


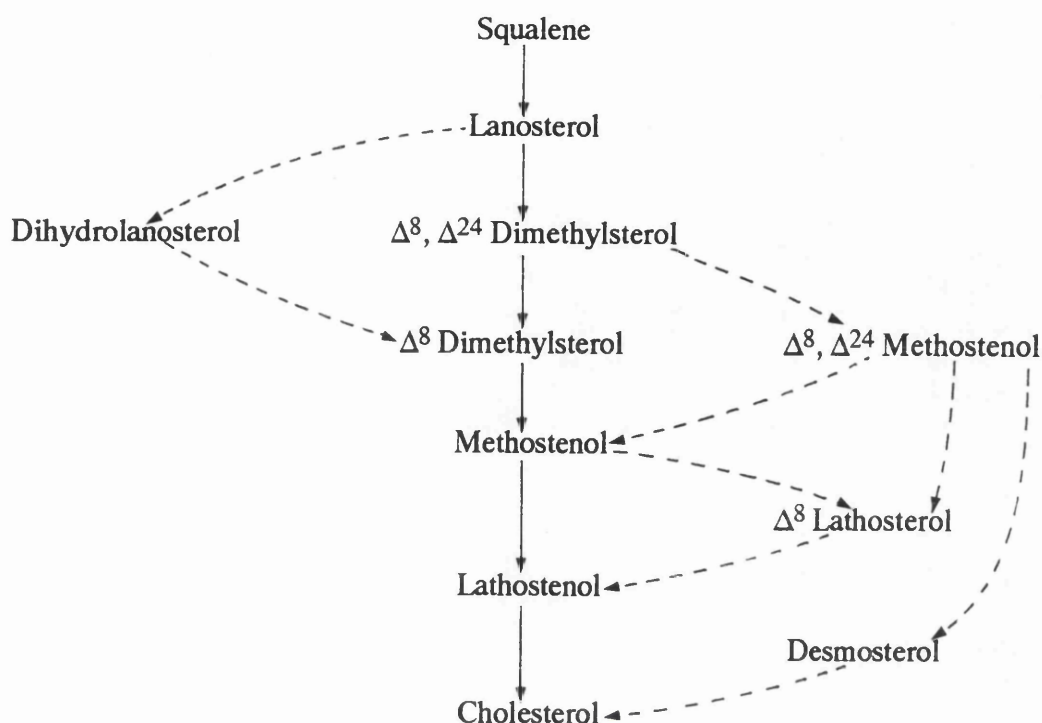
Figure 63. The Cholesterol Biosynthetic Pathway (Adapted from Stryer 1981)

Plasma mevalonic acid exhibits a diurnal variation (Parker *et al* 1982, Parker *et al* 1984), the control of which has yet to be fully elucidated. The measurement of serial plasma mevalonic acid levels in human subjects is interesting and may yield very useful data on the possible mechanisms controlling the diurnal cycle. However, such serial plasma sampling is difficult and impractical on all but an in-patient basis. Unlike other lipid soluble intermediates in sterol biosynthesis mevalonic acid is excreted in the urine (Kopito & Brunengraber 1980, Kopito *et al* 1982). 24 hour urinary mevalonic acid estimation has been shown to reflect the integrated plasma concentration throughout the 24 hour period (Parker *et al* 1984). The use of this assay in place of one-off plasma mevalonic acid levels obviates potential problems and allows ready comparison between or within subjects at different times (Parker *et al* 1982, Parker *et al* 1984).

This approach is considered to be a simpler and more practical way of indirectly assessing human cholesterol biosynthetic rates than conventional sterol balance studies (Pappu, Illingworth & Bacon 1989, Illingworth, Pappu & Gregg 1989). The effects of physiological and pharmacological modulation of cholesterol metabolism may therefore be assessed by 24 hour urinary mevalonic acid assays (Kopito *et al* 1982, Pappu & Illingworth 1989).

Serum levels of lathosterol, a late precursor of cholesterol (figure 64), have been shown to alter in the same direction as the expected rates of cholesterol synthesis with various manipulations including small bowel resection (Miettinen 1985) and cholestyramine and bile acid administration (Miettinen 1981). Further validation of the use of serum lathosterol as an index of cholesterol biosynthesis was obtained by correlating lathosterol levels with hepatic HMG CoA reductase activity (Bjorkhem *et al* 1987) and cholesterol balance studies (Kempen *et al* 1988). Additional corroboration was gained with the demonstration that this cholesterol precursor fell markedly in patients receiving HMG CoA reductase inhibitor therapy (Kempen *et al* 1988, Reihner *et al* 1990b, Elmberger *et al* 1991).

While there have been these reports of the effects of an HMG CoA reductase inhibitor on serum lathosterol, and others documenting changes in urinary mevalonate excretion with these drugs (Pappu & Illingworth 1989), these cholesterol precursors have never been determined simultaneously in the same subjects nor have there been any reports of the effects of the fibric acid derivatives on these indices of cholesterol biosynthesis. The latter is of particular interest as there is considerable controversy over the precise mechanism of cholesterol lowering with the fibrates.



**Figure 64.** Proposed Conversion of Squalene to Cholesterol in Humans. Major pathways are shown with solid arrows and minor or hypothetical pathways are shown with broken arrows (Redrawn from Bjorkhem *et al* 1987).

## 9.2 Study Protocols

I. Simvastatin. In 6 of the subjects recruited for the kinetic study described in chapter 3, plasma lathosterol and urinary mevalonate excretion were measured both at baseline and after 8 weeks therapy with simvastatin (20mg. d<sup>-1</sup>).

II. Fenofibrate. In 6 subjects who were recruited for a study to examine the effects of the fibric acid derivative, fenofibrate, on apo-LDL metabolism (results not reported here), plasma lathosterol and urinary mevalonate excretion were measured both at baseline and after 8 weeks therapy with fenofibrate (100mg t.i.d.).



In each study, the patients were maintained on their standard lipid lowering diet throughout. Just before starting drug therapy the patients collected a 24 h urine specimen, which was delivered to the laboratory immediately on completion, where it was aliquotted and frozen without delay for mevalonate estimation. At the same time a plasma sample was collected at 08.00h after a 14 h fast. The timing of this sample was strictly adhered to in order to minimise the effects of diurnal variation on plasma lathosterol levels. This procedure was repeated after 8 weeks of therapy with either drug. The control and drug phase samples for each patient were placed in the same assay run to minimise inter-assay variability. The plasma lathosterol estimations were also performed in duplicate.

### 9.3. Results I Simvastatin

The effects of simvastatin on the lipid profile of the 6 subjects studied are shown in table 33. There were highly significant falls in total and LDL-cholesterol as previously described (chapter 3) with no significant effect on triglyceride, VLDL, or HDL-cholesterol. The responses in urinary mevalonate were marked and uniform (figure 65) with all subjects showing a fall in this parameter of between 30 and 76% (mean 57%,  $p < 0.03$ ). Similarly, when plasma total lathosterol/cholesterol ratio was examined before and during therapy all subjects showed a fall in this parameter of between 20 and 61% (mean 49%,  $p < 0.01$ ) (table 33, figure 65).

### 9.4. Results II Fenofibrate

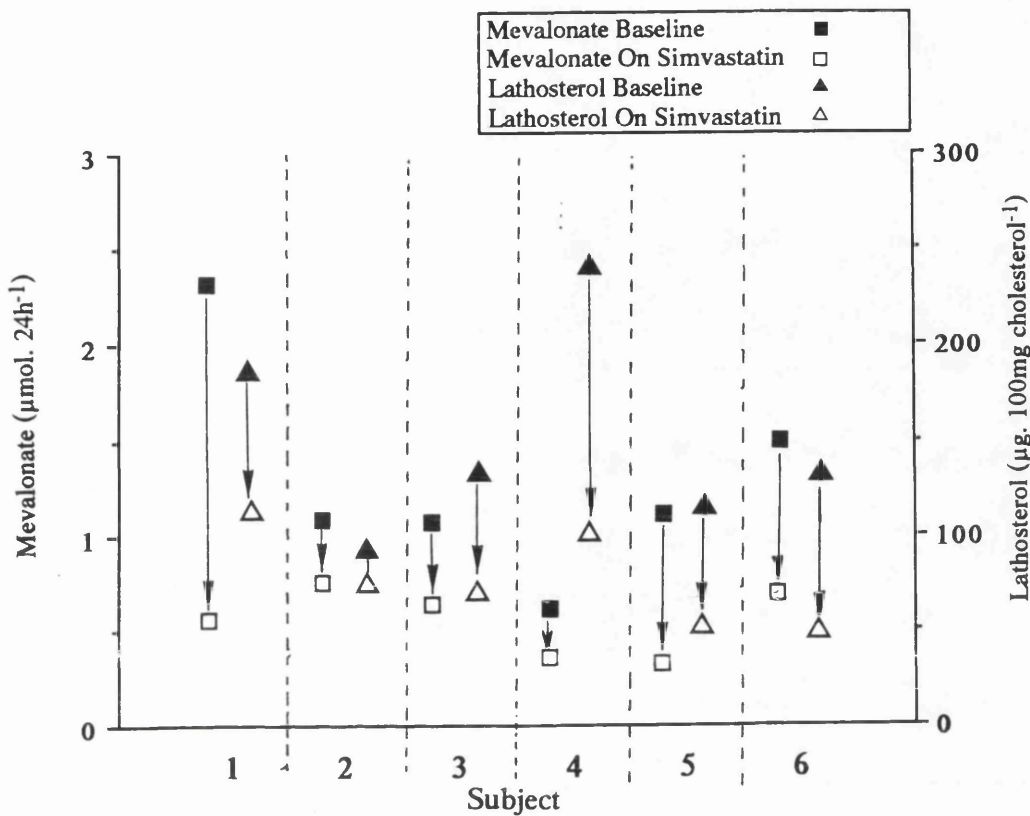
The effects of fenofibrate on the lipid profile of the 6 subjects studied are shown in table 34. There were significant falls in total, LDL and VLDL-cholesterol and in triglyceride while the increase in HDL-cholesterol did not reach statistical significance. The responses in urinary mevalonate were variable (figure 66) with two subjects showing a marked fall, two a marked increase and two staying approximately the same. The result of these variable changes was no overall change in the mean. Similarly, when plasma total lathosterol/cholesterol ratio was examined before and during therapy no significant change was observed (table 34, figure 66).

Table 33. Effects of Simvastatin Therapy on Lipids, Lipoproteins and Indices of Cholesterol Biosynthesis

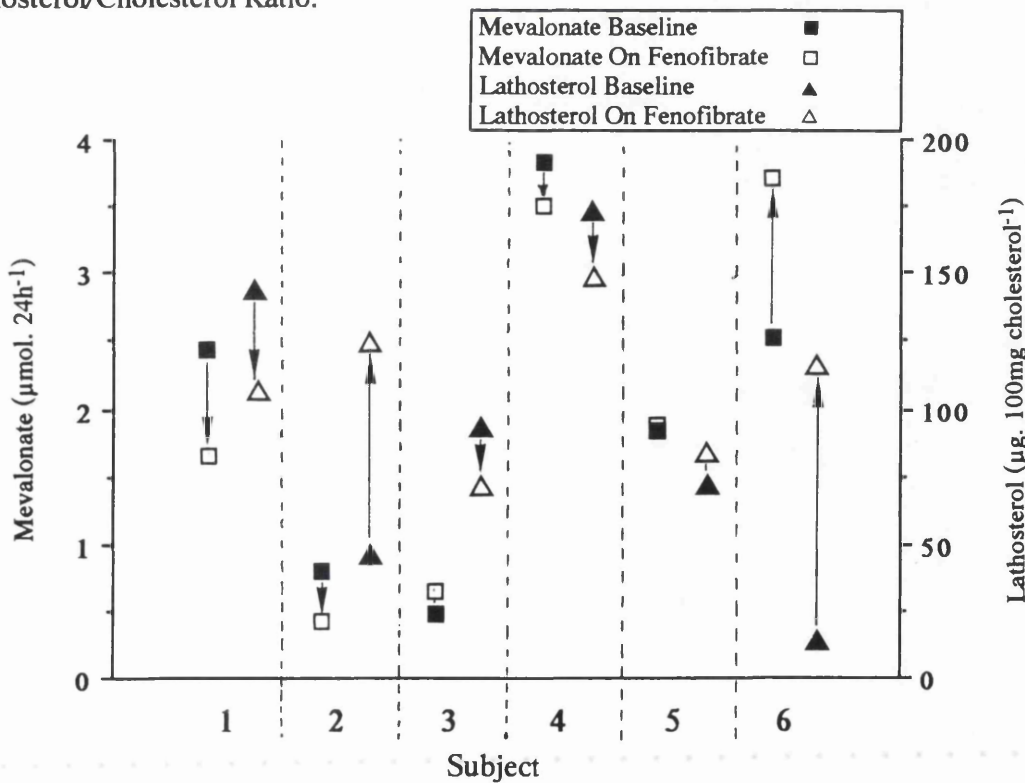
Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	Mevalonate	Lathosterol/Chol
			mmol. L <sup>-1</sup>			μmol.24h <sup>-1</sup>	μg. 100mg Chol <sup>-1</sup>
<i>Before Therapy</i>							
SIM 01A	7.5	1.20	0.35	5.10	1.60	2.35	189
SIM 02A	6.5	1.00	0.40	4.75	1.35	1.10	96
SIM 03A	9.6	3.45	2.40	6.25	0.90	1.10	134
SIM 04A	8.0	3.75	3.25	3.80	0.95	0.63	244
SIM 05A	7.2	2.25	0.75	5.00	1.40	1.12	116
SIM 06A	7.6	1.60	0.70	5.60	1.25	1.50	133
Mean (SEM)	7.7 (0.4)	2.21 (0.48)	1.31 (0.50)	5.08 (0.36)	1.24 (0.11)	1.30 (0.24)	152 (22)
<i>On Simvastatin</i>							
SIM 01B	5.0	1.20	0.60	2.95	1.45	0.57	114
SIM 02B	4.2	0.95	0.35	2.42	1.43	0.76	77
SIM 03B	6.3	2.15	1.40	4.25	0.90	0.65	72
SIM 04B	4.2	2.95	1.50	1.65	1.05	0.38	102
SIM 05B	5.5	1.80	0.75	3.20	1.55	0.32	53
SIM 06B	6.0	1.20	0.55	4.10	1.30	0.69	52
Mean (SEM)	5.2 (0.4)	1.71 (0.31)	0.86 (0.20)	3.10 (0.41)	1.28 (0.10)	0.56 (0.07)	78 (10)
p	<0.002	NS	NS	<0.001	NS	<0.03	<0.01

Table 34. Effects of Fenofibrate Therapy on Lipids, Lipoproteins and Indices of Cholesterol Biosynthesis

Subject	Cholesterol	Triglyceride	VLDL	LDL	HDL	Mevalonate	Lathosterol/Chol
			mmol. L <sup>-1</sup>			μmol.24h <sup>-1</sup>	μg. 100mg Chol <sup>-1</sup>
<i>Before Therapy</i>							
FEN 01A	8.00	1.74	0.75	5.70	1.55	2.45	160
FEN 02A	6.33	1.63	0.67	4.28	1.38	0.81	92
FEN 03A	8.20	1.30	0.35	6.45	1.38	0.48	124
FEN 04A	7.89	2.10	0.79	5.86	1.24	3.80	181
FEN 05A	8.50	1.59	0.61	6.58	1.28	1.83	110
FEN 06A	9.50	1.20	0.30	7.60	1.62	2.50	70
Mean (SEM)	8.07 (0.42)	1.59 0.13	0.58 (0.08)	6.08 (0.45)	1.41 (0.06)	1.98 (0.50)	123 (17)
<i>On Fenofibrate</i>							
FEN 01B	6.04	0.91	0.45	3.89	1.70	1.69	108
FEN 02B	5.33	1.10	0.31	3.62	1.39	0.42	126
FEN 03B	5.30	0.89	0.29	3.59	1.40	0.65	73
FEN 04B	5.54	1.19	0.45	3.71	1.38	3.55	149
FEN 05B	5.60	0.96	0.34	3.50	1.73	1.80	85
FEN 06B	7.26	0.88	0.34	5.34	1.59	3.68	117
Mean (SEM)	5.85 (0.30)	0.99 (0.05)	0.36 (0.03)	3.94 (0.29)	1.53 (0.07)	1.96 (0.57)	110 (11)
P	<0.002	<0.002	<0.03	<0.003	NS	NS	NS



**Figure 65.** Effects of Simvastatin on Urinary Mevalonate and Plasma Lathosterol/Cholesterol Ratio.



**Figure 66.** Effects of Fenofibrate on Urinary Mevalonate and Plasma Lathosterol/Cholesterol Ratio

## 9.5 Discussion

The use of simvastatin, a known inhibitor of cholesterol synthesis, in these studies has served to test the ability of the mevalonate and lathosterol assays to detect changes in cholesterol synthesis. Significant changes in both plasma lathosterol and urinary mevalonate excretion confirm previous findings of Pappu & Illingworth (1989), who noted a 34% fall in urinary mevalonate associated with lovastatin therapy. These workers also quoted a reference interval for urinary mevalonate excretion of 1.0-2.95  $\mu\text{mol. } 24\text{h}^{-1}$ . The range of baseline values recorded in the present studies was 0.48-3.80  $\mu\text{mol. } 24\text{h}^{-1}$ , which encompasses their interval and is therefore reasonably consistent in view of the relatively small number of measurements.

The main objective of this study was to assess the effects of a fibric acid derivative on cholesterol synthesis. A more detailed background to this question will be germane to the discussion that follows.

Besides their effects on triglyceride and apoB metabolism the fibrates are also reputed to have an inhibitory effect on hepatic cholesterol synthesis. This action is thought to be mediated through the inhibition of HMG CoA reductase, the rate limiting enzyme of cholesterol biosynthesis. Early work by Berndt and coworkers (1978) demonstrated both the inhibition of this enzyme by clofibrate and bezafibrate in rat liver microsomes *in vitro*, and its reduced activity in liver microsomes isolated from fibrate fed rats. Schneider *et al* (1985) have also demonstrated reduced activity of HMG CoA reductase in freshly isolated mononuclear cells from patients treated with fenofibrate, but in similar studies, using bezafibrate, Stange and colleagues (1991) report no drug induced effect on HMG CoA reductase activity. McNamara *et al* (1980) also observed no change in the rate of cholesterol synthesis in mononuclear cells freshly isolated from patients receiving clofibrate therapy. Other recent studies have demonstrated fibrate induced inhibition of the regulatory enzymes in cholesterol biosynthesis. Castillo *et al* (1990) showed reduced activity of the enzyme HMG CoA reductase *in vivo* and *in vitro* in chick liver in response to clofibrate, while Cosentini *et al* (1989) demonstrated in a total of 15 patients that bezafibrate reduced the incorporation of labelled acetate into non-saponifiable lipids in freshly isolated blood mononuclear cells. Again, however, there is some equivocation in the literature in that another fibrate, gemfibrozil, has been shown to increase hepatic sterol biosynthesis, albeit in rats (Maxwell *et al* 1983).

Published reports to date have, however, been criticised on a number of counts. Just as there are differences between rodents and humans in the hepatic toxicology of the fibrates, there also appear to be important species dissimilarities in the effect of the fibrates on the enzymes of cholesterol metabolism as documented by Stahlberg *et al* (1991). These workers report an approximately two-fold increase in HMG CoA reductase activity in human liver microsomes from bezafibrate treated individuals, in contrast to the reductions seen in other studies in animals. The message here is that work done in animal models may not be readily extrapolated to man.

Much of the published data have also been criticised either because of invalid methodology (Fears 1983), or the use of unrealistically high concentrations of drug *in vitro* (Newton & Krause 1986). The latter workers point out that many *in vitro* experiments may be flawed because the concentrations of fibrates used, greatly exceed potential *in vivo*, pharmacological levels. Similar levels of HMG CoA reductase inhibition were achieved by these workers when compactin was used as a positive control at *in vitro* levels 10,000 fold lower than that required of the fibrates. On this basis, Newton and Krause (1986) suggest that the observed inhibitory effect of the fibrates on HMG CoA reductase *in vitro* may represent a non-specific rather than a physiological effect at a specific, regulatory site on the enzyme. It is not appropriate, therefore, to class the fibrates with the specific HMG CoA reductase inhibitors or 'statins' even though there is some good evidence that they do indeed inhibit cholesterol synthesis, for they must effect this action by other means. Indirect effects of the fibrates on cholesterol biosynthesis have been proposed by Kleinman *et al* (1985). They have shown that the compositional changes in LDL induced by bezafibrate therapy enhances LDL receptor-mediated uptake of this lipoprotein and its ability to down-regulate LDL receptor activity and to suppress cholesterol synthesis in cultured fibroblasts. This enhanced delivery of sterol to cells may indirectly inhibit cholesterol synthesis in fibrate treated patients, in accord with the concept of Brown *et al* (1974), who found that cholesterol itself was the feedback suppressor of HMG CoA reductase activity in fibroblasts. Another indirect effect of the fibrates on the enzymes of cholesterol synthesis may be mediated by drug induced changes in membrane cholesterol content, and, in turn, membrane fluidity. Mitropoulos and Venkatesan (1985) describe possible mechanisms by which the enzyme HMG CoA reductase, a membrane bound protein, may be regulated by the cholesterol content of its supporting phospholipid bilayer. Needham *et al* (1985) have studied the effects of fibrate therapy on membrane fluidity and conclude that the drug clofibrate decreases the molar

cholesterol/phospholipid ratio of plasma membranes and that this environmental change alters the functioning of integral membrane proteins. Thus, fibrate induced changes in the membranes of the endoplasmic reticulum, where HMG CoA reductase is mainly located, may indirectly influence this enzyme's activity. While there has been some equivocation in the literature from *in vivo* and *in vitro* experiments, there is now emerging a consensus that the fibrates do indeed inhibit cholesterol synthesis in man but that they do so by some means other than the direct inhibition of the enzyme HMG CoA reductase. The present study, however, which is the only report of the effects of a fibrate on indirect indices of cholesterol synthesis does not support this hypothesis. The lack of a consistent lowering effect of fenofibrate on plasma lathosterol, or in the same subjects, urinary mevalonate excretion, suggests that this drug is not a cholesterol synthesis inhibitor in the same way that simvastatin obviously is. Further work in this area is clearly needed before we fully understand the nature of the mechanisms by which the fibrates exert their cholesterol lowering effect.

## 9.6. Further Considerations

Before leaving this topic there are two other areas that deserve some comment. These are alternative ways of measuring mevalonate and alternative uses of the mevalonate and lathosterol assays.

The mevalonate assay, while useful as a research tool is difficult to perform and is very expensive in terms of time reagents and consumables. Alternative ways to measure mevalonate have therefore been sought. The estimation of mevalonate in urine and plasma using gas chromatography-mass spectrometry (GC-MS) and selected ion monitoring has been described by Del Puppo *et al* (1988). This procedure gives results in the same range as those obtained with the radio-enzymatic assay reported here, but has been criticised by another team (Hoffman & Sweetman 1990) who considered the method unsuitable to quantify normal physiological samples reliably. Galli Kienle *et al* (1991) replied to these methodological criticisms, stating that they found the derivitisation technique used to be convenient and to yield consistent results. The controversy over the validity of the determination of mevalonate by GC-MS continued until Scoppola *et al* (1991) reported their modifications of the assay described by Del Puppo *et al* (1988). Using an electron-capturing bis(trifluoromethyl)benzyl derivative in a GC-ECMS system these workers reported improved specificity and sensitivity.

The mevalonic acid and lathosterol assays have recently gained a new clinical significance with the introduction of the HMG CoA reductase inhibitor class of hypolipidaemic drugs. The measurement of mevalonic acid in patients receiving such therapy has been suggested as a useful biochemical monitor in addition to lipoprotein levels (Kempen *et al* 1988, Pappu, *et al* 1989, Pappu & Illingworth 1989, Thompson *et al* 1989). Further applications of the mevalonate and lathosterol have also been identified in recent years. The autosomal recessive inborn error of metabolism, Mevalonic Aciduria is due to a deficiency of the enzyme mevalonic kinase (figure 63). First described by Berger *et al* (1985) and subsequently by Hoffman *et al* (1986) it is an extremely rare condition (only three cases are described in the literature), but its diagnosis is largely dependent on the assay of mevalonate in plasma or urine. Because it can produce severe neurological defects and failure to thrive with death in infancy this disease is suitable for prenatal diagnosis in affected families. Such a diagnosis is described by Hoffman *et al* (1986) where grossly elevated levels of mevalonate in maternal urine indicated that the fetus was affected. This condition is the only known inborn error of the cholesterol biosynthetic pathway and as such offers a unique possibility of greater understanding of this complex area.

More recently, Wolthers *et al* (1991) reported a new application of the lathosterol assay. Investigating the condition, cerebrotendinous xanthomatosis (CTX) (cholesterol 26-hydroxylase deficiency), they identified a significant difference in serum lathosterol concentrations between normals and untreated CTX patients. Furthermore, these workers noted that the elevated levels of lathosterol in CTX returned to normal with oral bile acid therapy. They concluded that serum lathosterol may be useful to monitor the treatment of these patients.

Recent investigations have found that several cellular proteins viz. growth-regulating p21<sup>ras</sup> proteins, encoded by *ras* proto-oncogenes and oncogenes, are isoprenylated and contain covalently bound farnesyl or geranyl-geranyl moieties, which anchor them to cell membranes (Goldstein & Brown 1990). Inhibition of mevalonate synthesis prevents farnesylation of these proteins and blocks cell growth. Therefore, the importance of products of the mevalonate pathway in the pathology of malignancy as well as that of atherosclerosis places these compounds at the centre of modern medicine. Further studies of this pathway, in part facilitated by assays such as described here, may yield new and exciting developments in both these spheres.



## 9.7. Conclusion

In conclusion, this short study has served to confirm the lipid lowering efficacy of simvastatin and fenofibrate in two groups of moderately hypercholesterolaemic subjects. With the measurement of plasma lathosterol and urinary mevalonate excretion, two established indirect indices of cholesterol biosynthesis, the inhibitory effect of simvastatin on this pathway has been demonstrated. The effects of fenofibrate have, on the other hand, failed to confirm that this drug exerts any consistent effect on the cholesterol biosynthetic pathway. Such new information may be used to re-evaluate the mechanism of action of the fibric acid derivatives. the execution of these studies has also served to establish two new and powerful assays side by side, which if early work is confirmed, may have much wider applications than originally thought.

## *Chapter 10 Conclusions*

As the area of light increases, so does the circumference of darkness.

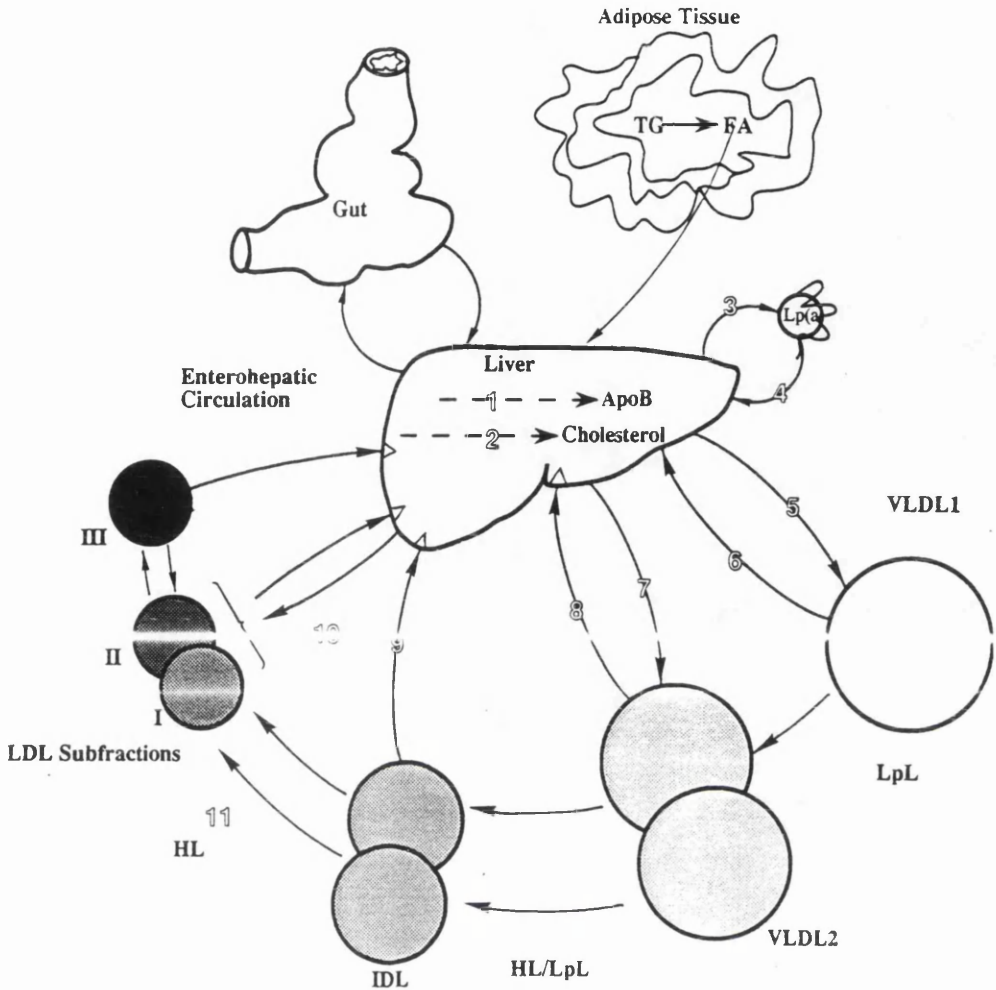
Albert Einstein.

### **10.1 Introduction**

As Einstein perceived, one of the principal products of scientific discovery is a realisation of what has yet to be uncovered. Most theses will have a collection of interesting conclusions but many will pose even more interesting questions. This thesis is no different. In chapter one, the dual aims of this work were set out: to investigate the pathophysiology of apoB metabolism and to test the effects of a range of lipid lowering drugs, singly and in combination, on this system. It is the purpose of this final chapter to assess the extent to which these aims have been satisfied and, in turn, the contributions that have been made to this field.

### **10.2 Pathophysiology of Lipoprotein Metabolism**

A schematic representation of apoB metabolism, which includes information derived from the present studies is illustrated in figure 67. What follows is a short discussion on the main findings, relating to the pathophysiology of the apoB containing lipoproteins, which are included either because they offer important new information or because they point in a new direction.



**Figure 67.** Schematic Representation of ApoB Metabolism. LpL=Lipoprotein Lipase, HL=Hepatic Lipase,  $\Delta$ =apoB/E receptor.

Total apoB synthesis (1) is relatively constant both in hyperlipidaemic states and after drug therapy. This parameter must be under different control from that of apoB/E receptor expression, cholesterol (2) and triglyceride synthesis. Lp(a) synthesis (3) and catabolism (4) were unaffected by drug therapy, which altered LDL levels, and are therefore placed separately from the other apoB containing lipoproteins. There is direct hepatic synthesis of VLDL<sub>1</sub> (5) and VLDL<sub>2</sub> (7), and direct clearance of these lipoproteins, mediated by the apoB/E receptor in the case of VLDL<sub>2</sub> (8) but not VLDL<sub>1</sub> (6). Clearance of IDL (9) is markedly affected by up-regulation of the apoB/E receptor and this appears to be one of the principal effects of those drugs which enhance receptor function. The direct synthesis of LDL (10) has been shown to be correlated with the plasma levels of LDL-I and II but not III. As such, direct synthetic pathways are shown only involving the larger and lighter subfractions, although receptor mediated clearance is probably involved in the catabolism of all three subfractions albeit in differing proportions. The final step in VLDL-derived LDL synthesis, i.e. the conversion of IDL to LDL (11) is mediated by hepatic lipase. Inhibition of this enzyme by various means including acipimox therapy results in decreased LDL synthesis. The question of which LDL subfraction are specifically derived from the delipidation cascade and the nature of their interconversions has not been the subject of this thesis and is not shown in the diagram.

### 10.2.1. The Nature of LDL Production

The rate and nature of LDL generated from VLDL may be perturbed pharmacologically, as has been shown in the preceeding chapters. This has important implications for the potential atherogenicity of LDL. A more contentious issue, which we are forced to address because of its quantitative importance, is that of direct LDL synthesis. A feature of the model of apoB metabolism used throughout this work is a direct input pathway at the level of LDL (figure 14). This was required to explain the observed LDL apoB mass, which is unaccounted for by that entering the LDL interval from the delipidation cascade, as detected using VLDL<sub>1</sub> and VLDL<sub>2</sub> tracers. As discussed in chapter 3, the nature of the physiological manifestation that explains this mathematical requirement is controversial. The consistent need for such a pathway in these studies may be offered as supportive evidence for its existence, although the nature of the present studies do not allow us to say whether this LDL is derived *de novo* or from a rapidly metabolised LDL precursor pool. All that can be said with surety is that there is an LDL apoB pool of variable size in almost all individuals, which is not trace labelled by the injection of the radiolabelled precursors, VLDL<sub>1</sub> and VLDL<sub>2</sub>. Of course, it may also be argued that there is some consistent error in the methodology used here, and that there would be no need to invoke the direct input of LDL if all LDL precursors were trace labelled equally.

It is not possible to resolve this question simply by re-analysing the data with alternative models of apoB metabolism, because a large number of equally plausible answers may be obtained by this manipulative strategy. The model used must be as simple and as physiologically pertinent as possible. The model must also explain the data obtained in all patients, in all situations. The current model has been developed over a number of years and has only been changed or expanded to accommodate new data when absolutely necessary. As such, although apparently complex, this model does satisfy the above criteria, in being the simplest solution possible for all circumstances.

The resolution of this issue will need a fundamentally different approach. Such a new technology is already available in the form of stable isotope labelled precursor turnovers. In these studies, the infusion of deuterated or <sup>15</sup>N-labelled amino acids, results in the stable isotopic enrichment of all proteins synthesised during the infusion period. If these proteins can be isolated in a pure form then

a direct measure of their fractional synthetic rate (FSR) can be made. With radioisotopic studies, such as described in this thesis, all estimates of synthetic parameters are derived indirectly from directly measured catabolic parameters using the assumptions of steady state kinetics. With stable isotope technology, on the other hand, synthetic parameters are measured directly, and it should be possible not only to demonstrate if LDL is produced *de novo* as well as by the delipidation of VLDL, but also which LDL subfractions are involved.

### 10.2.2. LDL Subfractions

An important observation in the present study was the correlation between LDL direct synthesis and the LDL subfractions I+II, but not LDL III. Although this correlation was significant only in those subjects recruited for the simvastatin study described in chapter 3, there was a similar trend in the other groups. Using the stable isotope technology described above the differential synthetic rates, if any, in the LDL subfractions may be observed. Such experiments, to test the hypothesis generated by the present work, that LDL direct synthesis accounts for a portion of the LDL I+II but not LDL III plasma concentrations, are currently underway in the Institute of Biochemistry at Glasgow Royal Infirmary.

The model of apoB metabolism used (figure 14) does embrace the concept of LDL heterogeneity by having two LDL compartments (10 and 11). With recent findings, including those reported here, this LDL subsystem will have to be re-evaluated and possibly redefined to allow for the three subfractions of LDL consistently observed in those patients studied. It is not known as yet, whether the three subfractions, although structurally distinct are metabolically dissimilar. The suggestion from the present work is that small dense LDL-III may be metabolically distinct from the larger more buoyant LDL I and II, while the latter two may behave in a similar fashion. Little is known about the potential interconversions of the LDL subfractions but this will be readily studied using radiolabelled LDL subfraction turnovers. Such studies are again currently underway and their results should provide valuable new information on the intravascular remodelling of these lipoproteins as well as their metabolic fates. With this new data an improved model of apo-LDL metabolism encompassing the observed heterogeneity in LDL will be constructed.

### 10.2.3. Lp(a)

The lack of any effect on plasma Lp(a) levels by the lipid lowering drugs studied here is consistent with many other reports. In view of the report from Carlson *et al* (1989) of the Lp(a) lowering observed with nicotinic acid therapy, it is particularly interesting to note the inability of the nicotinic acid analogue, acipimox, to lower Lp(a). This observation has recently been corroborated by Seed and her colleagues (1992) who reported a 32% fall in Lp(a) with nicotinic acid therapy ( $3\text{g. d}^{-1}$ ) but no change with acipimox ( $1000\text{mg. d}^{-1}$ ). Despite the similarities in the mechanisms of action of these two closely related drugs there is clearly a marked difference in their effects on Lp(a). Seed *et al* (1992) suggest that the Lp(a) lowering observed with nicotinic acid is due to a decrease in the synthesis of the lipoprotein rather than any change in its rate of clearance. Morrisett *et al* (1992) recently reported the results of kinetic studies of apo(a) using deuterated leucine. With this use of stable isotope labelled precursors a direct assessment of the synthetic rate of the endogenously labelled protein may be made. Morrisett *et al* (1992) showed that the FSR of apo(a) in Lp(a) was similar to that of apoB in Lp(a). The latter, however, was markedly lower than the FSR of apoB in LDL and these workers concluded that there must be two separate apoB pools for LDL and Lp(a) production. In addition they noted a strong negative correlation ( $r=-0.914$ ) between the apo(a) FSR and the plasma triglyceride level. This new information would suggest that the Lp(a) lowering facility of nicotinic acid is unrelated to its effects on triglyceride metabolism, as it would be expected from these findings that a drug such as nicotinic acid, which causes marked falls in plasma triglyceride would lead to enhanced Lp(a) production. However, the opposite effect is observed. With the information currently available it is not possible to offer a good explanation for this differential effect on Lp(a) by two apparently very similar drugs. With further studies on Lp(a) metabolism, particularly using stable isotope technology we may be able to dissect out the subtle differences in action of these drugs. As a corollary, further direct investigation into the mechanisms of action of nicotinic acid as compared with acipimox may also offer new insight into the metabolic control of plasma Lp(a) levels. As this lipoprotein is being viewed increasingly as an important therapeutic target, the design of competent new Lp(a) lowering drugs will largely depend on advances in this area.

#### **10.2.4. The Role of the Apo B/E Receptor**

By using drugs as stimulants of the apoB/E receptor, such as the HMG CoA reductase inhibitor, simvastatin, it has been possible to observe the changes in the apoB containing lipoproteins resulting from enhanced receptor activity. Increased clearance of LDL apoB, which was previously thought to be the principal result of increased apoB/E receptor expression was not consistently observed. Much more evident was the enhanced clearance of the LDL precursor, IDL apoB. This is an important finding drawing attention to the true nature of the apoB/E receptor, whose original name, the LDL receptor, reveals only part of the truth.

#### **10.2.5. Metabolic Heterogeneity and the Applicability of the Present Studies**

The marked variation in kinetic parameters between subjects presented a significant problem in the analyses of these data. The subjects studied here were deliberately chosen to represent the most commonly treated group of dyslipidaemics viz. those with primary moderate hypercholesterolaemia. Under this title there must clearly exist a whole range of metabolic defects contributing to the patients' hyperlipidaemia. This underlying heterogeneity presents problems when trying to study apolipoprotein kinetics. These studies are complex and labour intensive and pragmatic constraints usually confine the sample size to between 5 and 10 patients. One solution to this problem would be to select from this population of primary moderate hypercholesterolaemic subjects a group fully characterised for a specific set of genetic defects or phenotyped in a much more detailed manner. While this strategy would obviate much of the underlying heterogeneity observed in the present studies it could be argued that the results would be applicable only to that specific defect and not to the population as a whole, just as the findings both in terms of baseline kinetics and responses to drugs in those with FH are clearly inapplicable to the wider population.

### **10.3 Mechanism of Action of Lipid Lowering Drugs**

The second main aim of this study was to obtain new information on the mechanisms of action of examples of the main classes of lipid lowering drugs. The findings have already been discussed in the individual chapters and are now

summarised in figure 68. In the following sections the most relevant new findings are reiterated with suggested future possibilities.

### 10.3.1. Simvastatin

The most important finding here was the enhanced clearance of LDL precursors, VLDL<sub>2</sub> and IDL resulting in reduced throughput of apoB into the LDL interval. This drug induced effect on LDL synthesis rather than LDL clearance was surprising in view of the proposed mechanism of action of the statins i.e. enhanced apoB/E receptor function. However, with closer analysis of the literature it becomes clear that the LDL lowering facility of the statins can only be completely explained by increased LDL clearance in those individuals with a recognised defect of this kinetic parameter i.e. FH heterozygotes. The importance of this statin induced effect on apoB metabolism is the removal from the circulation of the more atherogenic lipoproteins. Such an effect should have important consequences in the prevention of CHD.

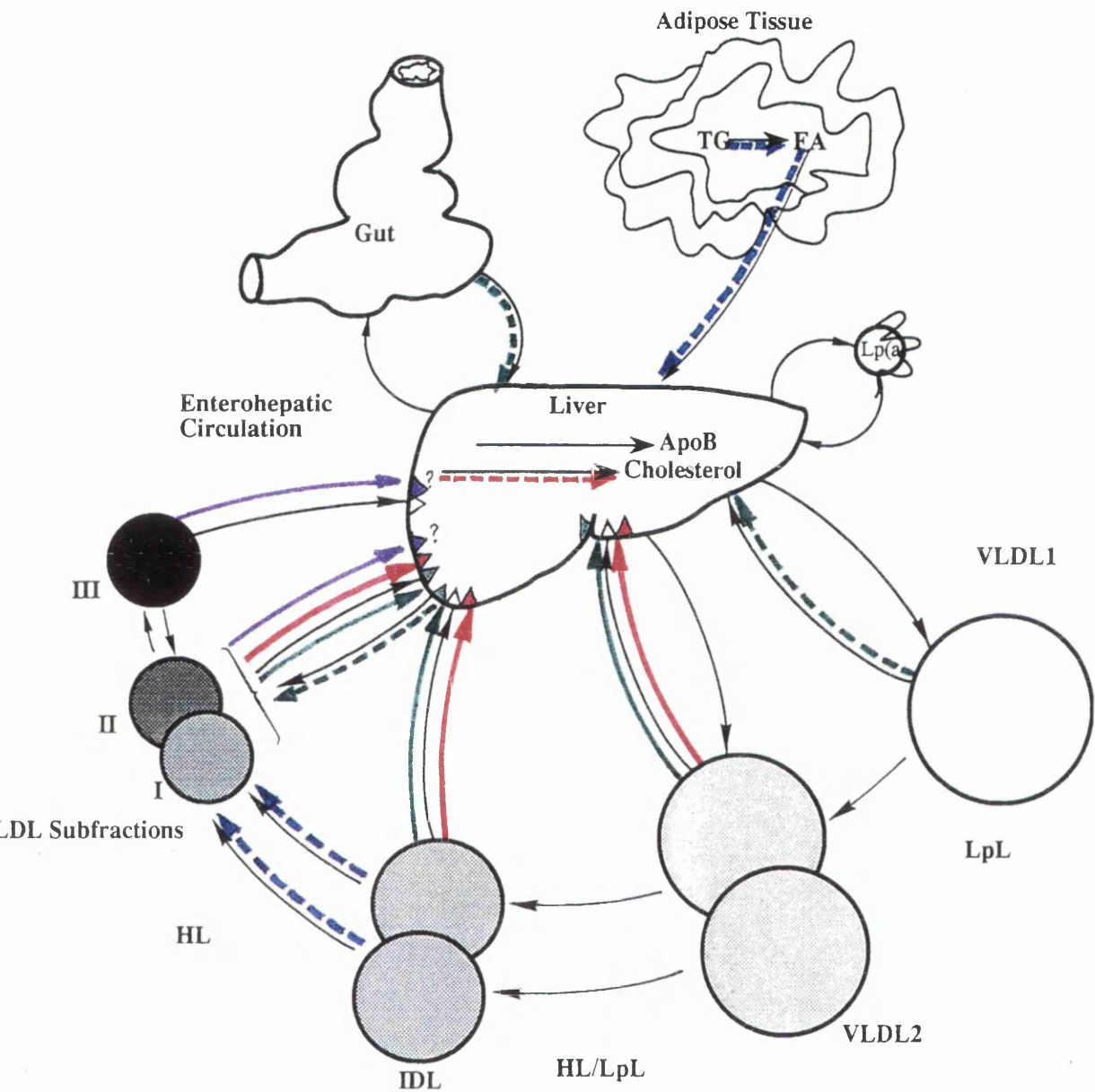
### 10.3.2. Colestipol

By studying the kinetics of the apoB containing lipoproteins a mechanism for the triglyceride raising effects of the bile acid sequestrant resins has been proposed. The marked reduction in the direct catabolism of VLDL<sub>1</sub> largely explains the increase in VLDL-cholesterol and plasma triglyceride observed with colestipol and its sister compound cholestyramine. With the renewed interest in the atherogenic potential of triglyceride rich lipoproteins this effect of a commonly used lipid lowering drug, albeit in conjunction with a lowering of LDL-cholesterol, may be considered disadvantageous. In the study of combined colestipol and simvastatin therapy (chapter 7) it was particularly of interest to note the complete reversal of this colestipol induced effect on VLDL metabolism when simvastatin was added to the regimen. Also in this study the beneficial effects on HDL-cholesterol were of note re-affirming the importance of this combination as an effective anti-atherosclerotic regimen.

### 10.3.3. Acipimox

The study of the nicotinic acid analogue acipimox provided the opportunity to examine a drug with diverse effects on opposite ends of the delipidation cascade. Inhibition of the synthesis of triglyceride-rich particles partly





**Figure 68.** Effects of Lipid Lowering Drugs on ApoB Metabolism. Baseline metabolism is shown in black, with the effects of simvastatin in red, colestipol in green, acipimox in blue and the fibrates in purple. Broken lines illustrate inhibition of a pathway, while solid lines show stimulation.

contributed to the fall in LDL by reducing the availability of lipoprotein precursors. However, acipimox also reduced the direct input of LDL and reduced the IDL to LDL apoB transfer. The latter is attributed to the known inhibitory effect of acipimox on hepatic lipase activity. These multiple effects on the metabolism of apoB containing lipoproteins serve to normalise not only an oversynthesis of LDL particles but also to reduce the circulating mass of triglyceride-rich particles, which as described above are considered to make a significant contribution to the the risk of atherogenesis.

#### 10.3.4. Ciprofibrate and Fenofibrate

By choosing specifically to study the effects of a fibrate on receptor mediated clearance of apo-LDL it was possible to confirm that the LDL-cholesterol lowering of ciprofibrate was indeed due to enhanced clearance of LDL by the high affinity receptor mediated route. It was also possible by studying the LDL subfraction changes associated with drug therapy to suggest that this kinetic effect may not simply be due to enhanced apoB/E receptor expression but may instead be due to the synthesis of more receptor-active LDL species. These proposed ligand changes were not supported by simple LDL compositional changes but were supported by significant changes in the LDL subfraction profile.

Another important finding in the present work was the lack of effect of the fibric acid derivative, fenofibrate on cholesterol biosynthesis, indirectly assessed by measurement of plasma lathosterol and urinary mevalonate excretion. This finding offers important human *in vivo* evidence in support of the hypothesis that the fibrates have no effect on HMG CoA reductase activity. This hypothesis has received an increasing body of supportive evidence from *in vitro* studies, most recently by Qin *et al* (1992). These workers reported a complete lack of effect of ciprofibrate on HMG CoA reductase or LDL receptor activity, or the gene expression of these two proteins in a human intestinal cell line, CaCo2. The present study is the first report of the effects of a fibrate on these indirect indices of cholesterol biosynthesis and highlights the utility of these assays in tandem as an important tool for the evaluation and investigation of new lipid lowering drugs that purport to have an effect on the cholesterol biosynthetic pathway. Such drugs to be investigated in the future might include the recently reported squalene synthase inhibitor (McCarthy *et al* 1992) and the new second generation HMG CoA reductase inhibitor BA W 6228 (Bischoff & Petzinna 1992).

#### 10.4. Full Circle

This thesis began with the words of an ancient physician, Hippocrates. While he would surely be fascinated, not to say beguiled, by the advances made in medicine in the last two millenia, he would be dismayed if we, as clinicians and scientists, were to forget his simple yet far reaching guidelines of medical care, placing the patient at the centre of all things. If this thesis has some merit it will be in advancing our understanding of an area of clinically relevant physiology, that of lipoprotein metabolism, and in placing the use of the lipid lowering drugs on a firmer theoretical footing. This is done first and foremost to help patients and to assist others in this simple yet all too often forgotten goal.

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## Appendix 1

### Suppliers of Reagents, Equipment and Software.

#### Amersham International plc

*Amersham Place, Little Chalfont, Amersham, Bucks. HP7 9NA, UK.*

Na [<sup>131</sup>I]

Na [<sup>125</sup>I].

[<sup>14</sup>C]-5-phosphomevalonic acid.

#### Amicon Ltd.

*Upper Mill, Stonehouse, Glos. GL10 2BJ, UK.*

Chromatography column

Ultrafiltration membrane (Diaflo)

Red Sepharose (Amicon Matrix Gel Red A)

#### Baker Instruments Ltd

*Rusham Park, Whitehall Lane, Egham, Surrey, TW20 9NW, UK.*

Encore Chemistry System

Centrifi Chem®

#### Baxter Healthcare Ltd

*8 Tollpark Place, Wardpark East, Cumbernauld G68 0LN, UK.*

0.9% sodium chloride intravenous infusion BP

#### Beckman Instruments, Spinco Division

*Beckman Instruments (UK) Ltd Analytical Sales and Service Operation*

*Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL, UK.*

L8-60M ultracentrifuge

Prep UV Scanner

SW 40 rotor

Ultra-Clear™ centrifuge tubes

Polycarbonate centrifuge bottles and caps

Silicone vacuum grease

L8-70 ultracentrifuge

AnF rotor

Ti 60 rotor

Ti 50.4 rotor

Spinkote

Tube Slicer

#### Becton Dickinson

*Dublin, Eire.*

Microlance® 21G Needles

Syringes

#### BDH Laboratory Supplies

*McQuilkin and Co., 21 Polmadie Avenue, Glasgow G5 0BB, UK.*

Triethylamine

Folin-Ciocalteu Reagent

#### Bio-Rad Laboratories Ltd

*Bio-Rad House, Marylands Avenue, Hemel Hempstead, Herts. HP2 7TD, UK.*

Bio-gel HT hydroxylapatite

Econo-columns

AG® 1-X8 200-400 mesh chloride form

#### Boehringer Mannheim GmbH

*Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd*

*Bell Lane, Lewes, East Sussex BN7 1LG, UK.*

ATP (MV 602)

Kit No 310328 (free cholesterol)

Kit No. 704121 (cholesterol)

Hitachi 704 automatic analyser.

Kit No. 69184 (phospholipid)

Kit No. 704113 (triglyceride).

#### Bristol Myers Squibb

*Squibb House, 141-149 Staines Rd, Hounslow, Middx TW3 3JA, UK.*

Cholestyramine

#### Chrompack Ltd

*Unit 4, Indescon Crt, Millharbour, London, E14 9TN, UK.*

CP 9000 Gas chromatograph

WCOT 50m x 0.25 capillary column

Deriva-Sil®

PCI integration software package

**Claris™**

440 Clyde Ave, Mountain View, CA, USA.  
MacDraw II

**Cricket Software Inc**

40 Valley Stream, Malvern, PA, USA.  
Cricket Graph Version 1.3.1

Statworks Version 1.2

**Dynatech Laboratories Ltd**

Daux Road, Billingshurst, West Sussex, RH14 95J, UK.  
MR5000 microtitre plate reader

**Eastman Fine Chemicals**

Eastman Kodak Co, Rochester, NY, USA.  
Decyl-sodium sulphate

**Farmitalia Carlo Erba Ltd**

Italia House, 23 Grosvenor Rd, St Albans, AL1 3AW, UK.  
Acipimox

**Fluka Chemical Ltd**

Peakdale Rd, Glossop, Derbyshire, SK13 9XE, UK.  
1,2 Cyclohexanedione

**Fournier**

Centre de Recherches de Daix, 50 rue de Dijon, Daix, 21121 Fontaine les Dijon, France.  
Fenofibrate

**Gelman Sciences Ltd**

Brackmill Business Park, Caswell Road, Northampton NN4 0ES, UK.  
Acrodisc filters 0.22 and 0.45 µm

**Haemonetics Corporation**

400 Wood Rd, Braintree, MA, USA.  
Haemonetics plasma collection system (PCS)

**Innogenetics SA**

Kronenburgstraat 45, B 2000 Antwerp, Belgium.  
Lp(a) Kit

**Isolab**

Advanced Laboratory Techniques, 11 Bounds Oak Way, Southborough, Tunbridge Wells, Kent, TN4 0UB, UK.  
Gradient maker

**Merck, Sharp and Dohme Ltd**

Hertford Rd, Hoddesdon, Herts. EN11 9BU, UK.  
Simvastatin

**Microsoft Corporation**

1 Microsoft Way, Redmond, WA, USA.  
MS Word Version 4.0

**MSE**

Fisons Instruments, Sussex Manor Park, Gatwick Rd, Crawley, Sussex, RH10 2QQ, UK.  
Mistral 4L refrigerated centrifuge.

**New England Nuclear**

NEN Research Products, Wedwood Way, Stevenage Herts. SG1 4QN, UK.  
<sup>32</sup>P-ATP.

**Paar Scientific Ltd***594 Kingston Road, Raynes Park, London SW20 8DN, UK.*

Digital densitometer DMA 35

**Packard Instrument Co.***Canberra Packard Ltd, Brook House, 14 Station Road, Pangbourne, Berks. RG8 7DT, UK.*

Ultima Gold scintillation fluid

Auto gamma 500 C

Super polyethylene vials (20mL)

Auto  $\beta$  counter**Pharmacia AB***Pharmacia Ltd, Pharmacia LKB Biotechnology Division, Midsummer Boulevard, Central Milton Keynes, Bucks. MK9 3HP, UK.*

Sephadex G-25 (PD10 columns)

**Resource Facility for Kinetic Analysis***Center for Bioengineering, FL-20, University of Washington, Seattle, WA 98195, USA.*

SAAM 29

SAAM 30

**Sanofi-Winthrop***Onslow St, Guildford, Surrey, GU1 4YS, UK.*

Ciprofibrate

**Scotlab***Kirkshaws Rd, Coatbridge, ML5 8AD, UK.*

Plexiglas shielding

**Sigma***Sigma Chemical Co. Ltd, Fancy Road, Poole, Dorset BH17 7TG, UK.*

Iodine monochloride (ICI)

Dithiothreitol (DTT)

Trishydroxymethylaminomethane (TRIS)

Tetramethylurea (TMU)

Protamine Sulphate

5 $\alpha$ -cholestane

Adenosine triphosphate (ATP)

Mevalonolactone

Phosphoenol pyruvate (PEP)

Pyruvate kinase (PK)

 $\beta$  NADH

Lactate dehydrogenase (LDH)

**Spectrapor***Orme Technology, PO Box 3, Stakehill Industrial Park, Middleton, Manchester, M24 2RH, UK.*

Dialysis tubing

**Sterilin UK***McQuilkin and Co., 21 Polmadie Avenue, Glasgow G5 0BB, UK.*

96 well microtitre plates

**Technicon (Ireland) Ltd.***Swords Co. Dublin, Eire.*

12 channel Peristaltic pump

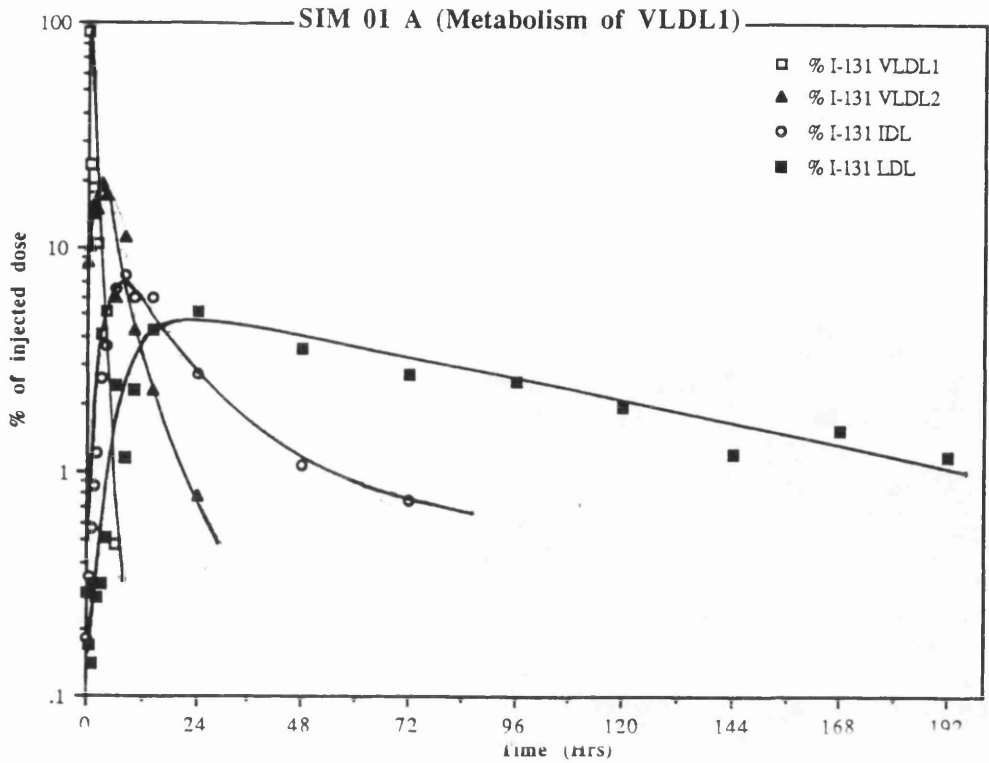
**Upjohn Ltd***Fleming Way, Crawley, West Sussex, RH10 2NJ, UK.*

Colestipol

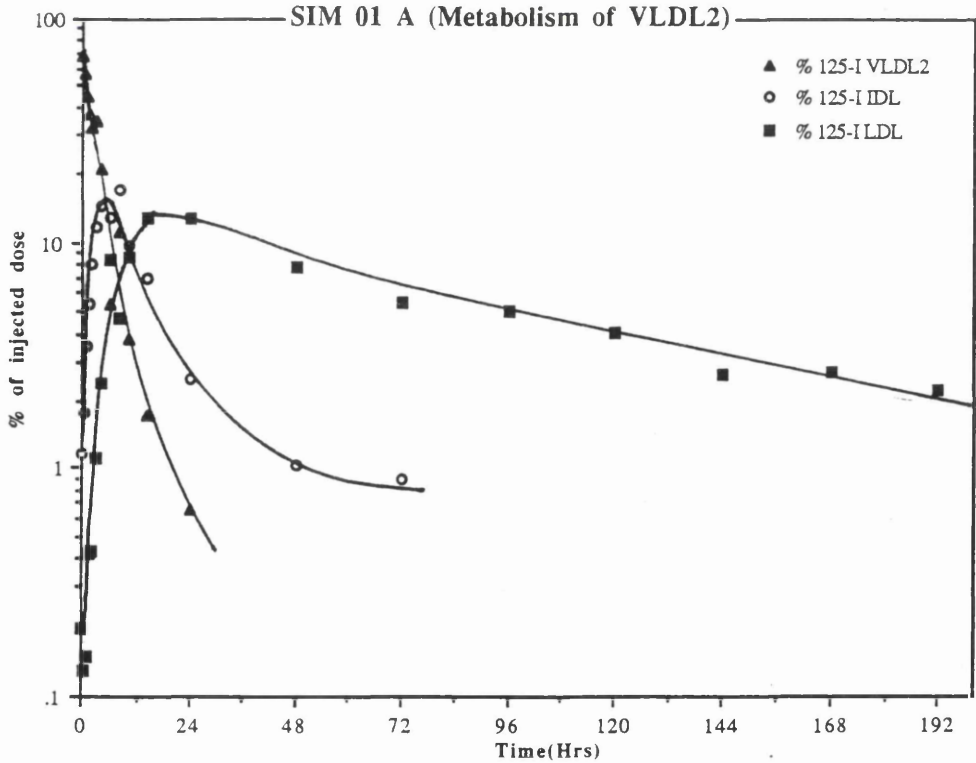
**Viggo AB***Helsingborg, Sweden.*

Venflon®2 intravenous cannulae

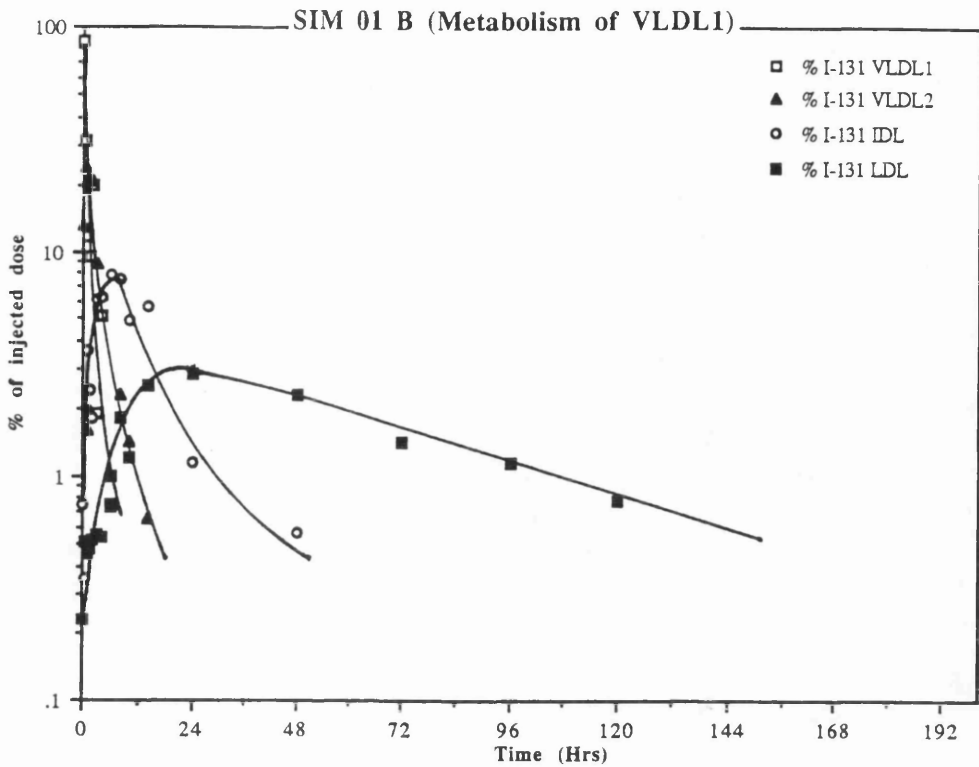
*Appendix 2*



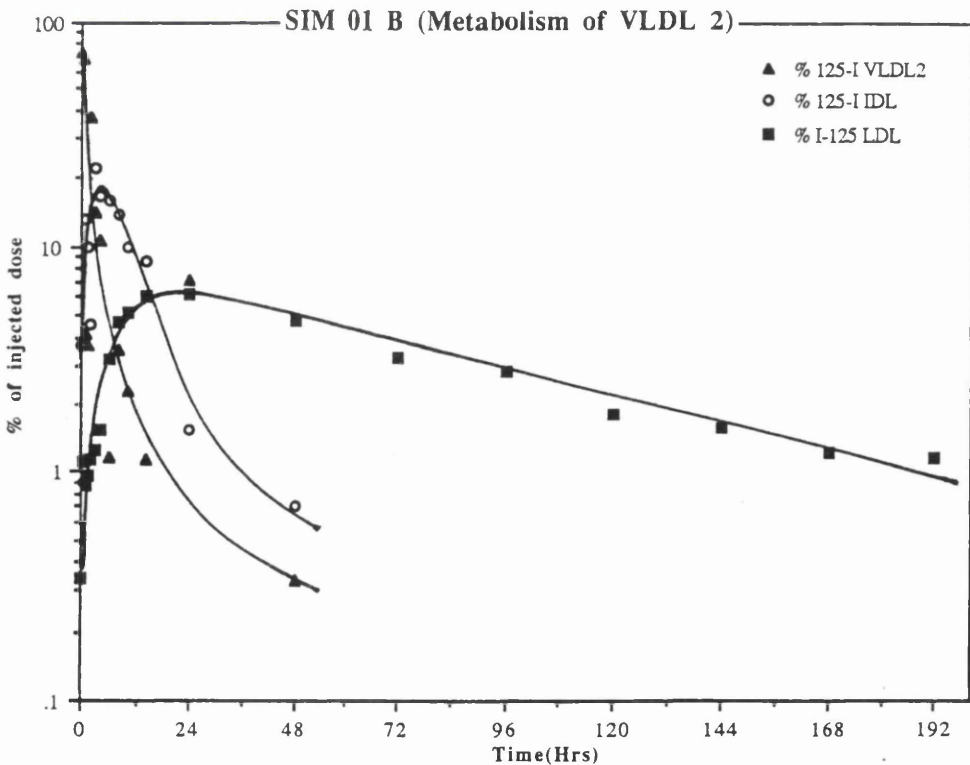
**Figure A-1.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



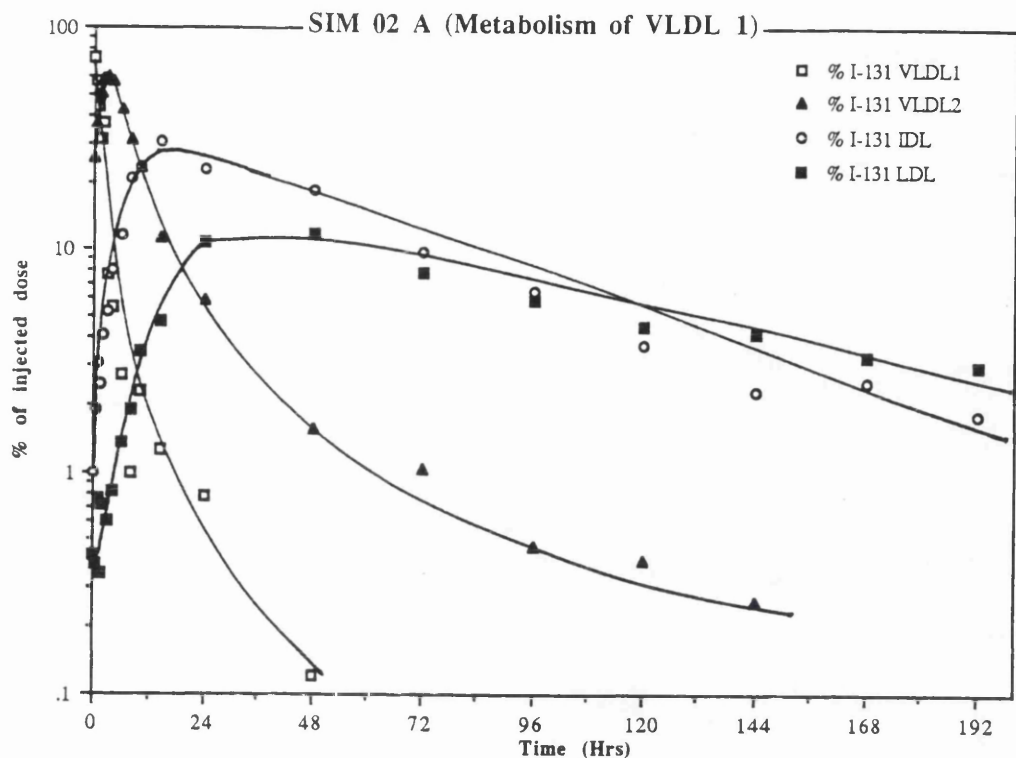
**Figure A-2.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



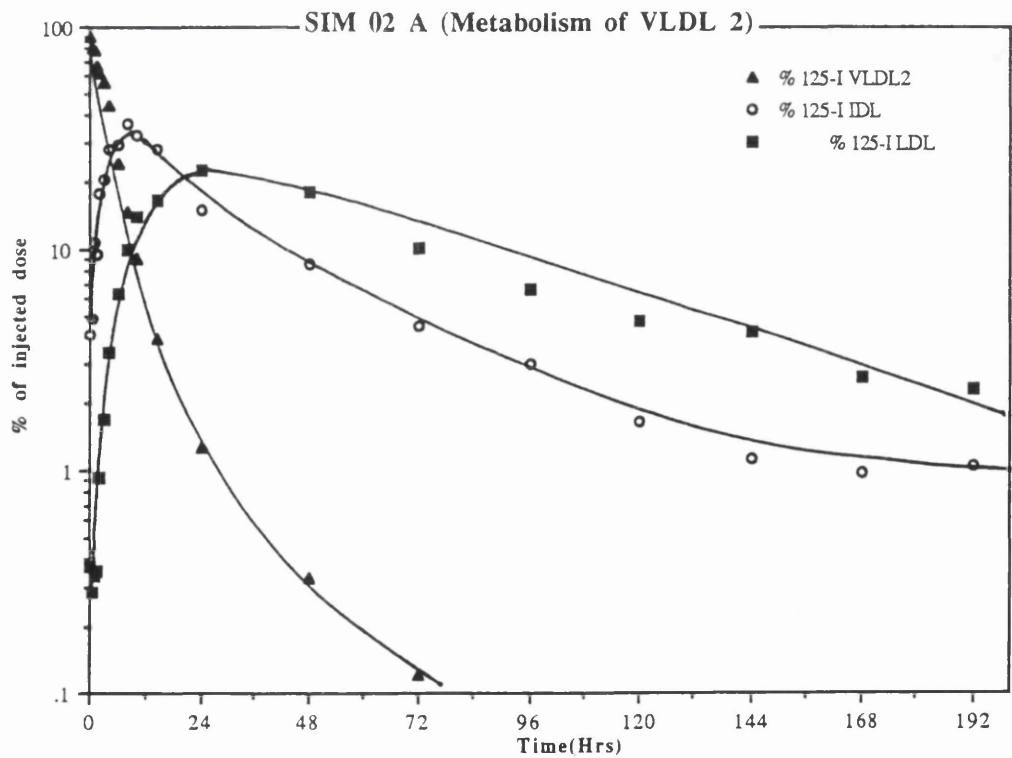
**Figure A-3.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-4.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

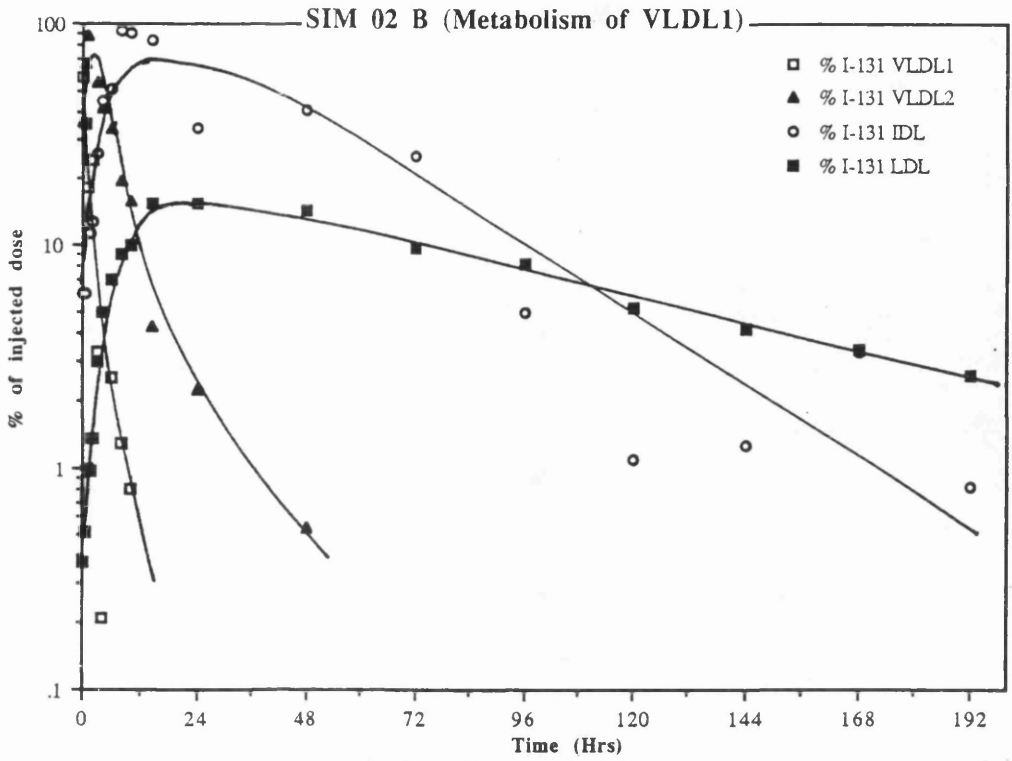


**Figure A-5.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

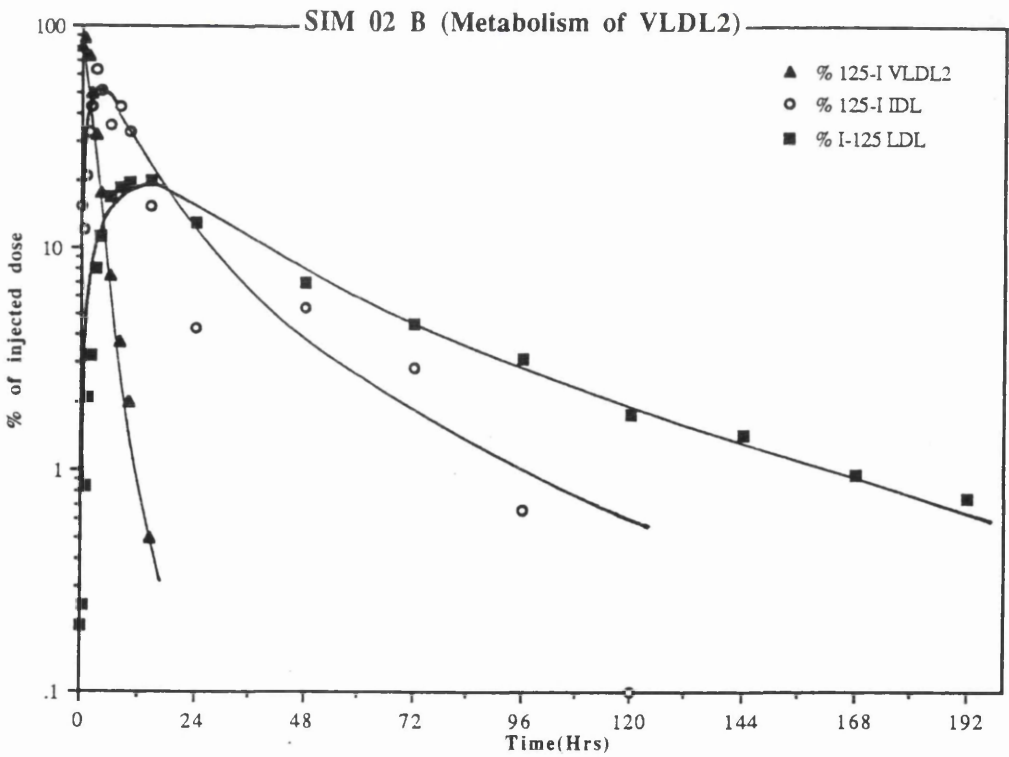


**Figure A-6.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

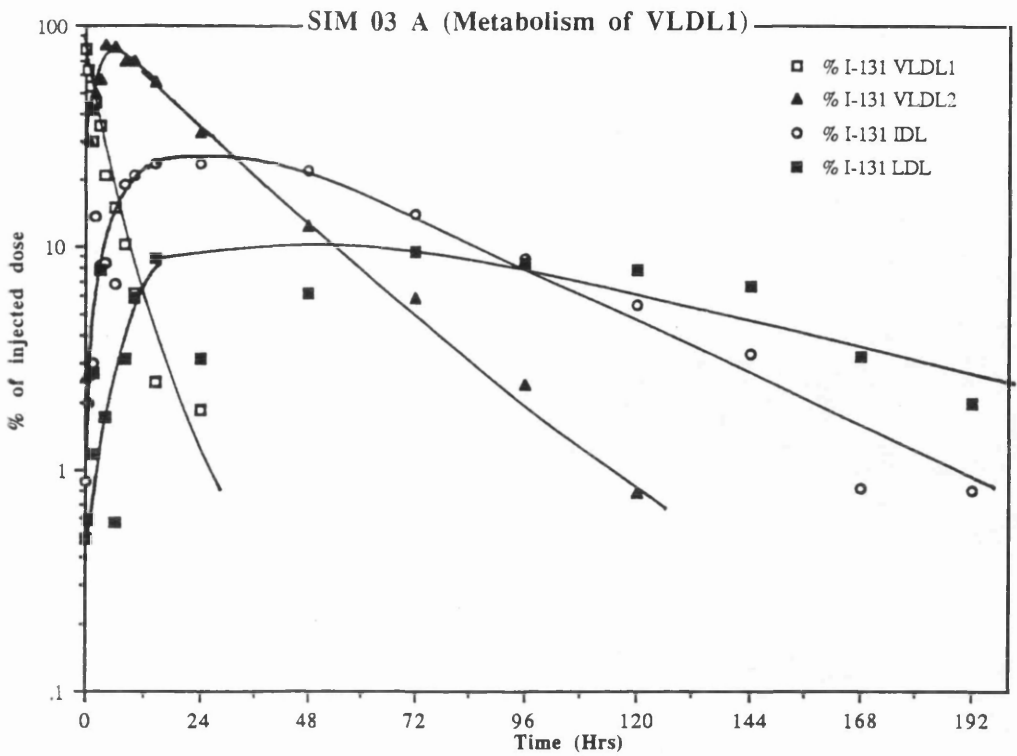




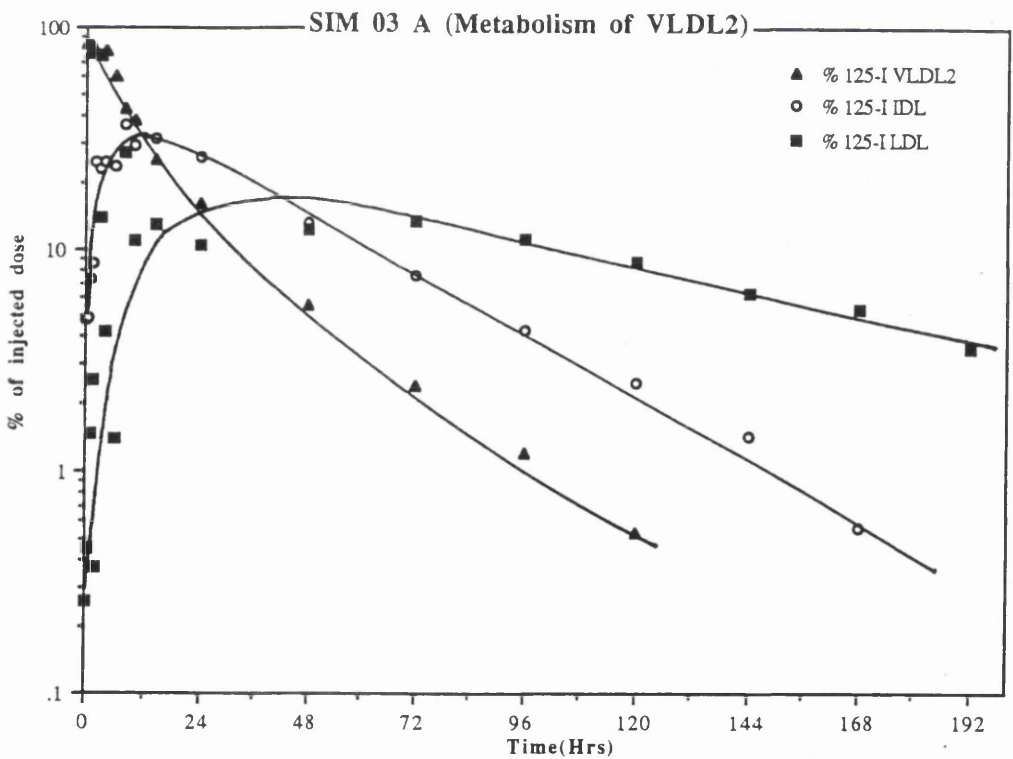
**Figure A-7.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



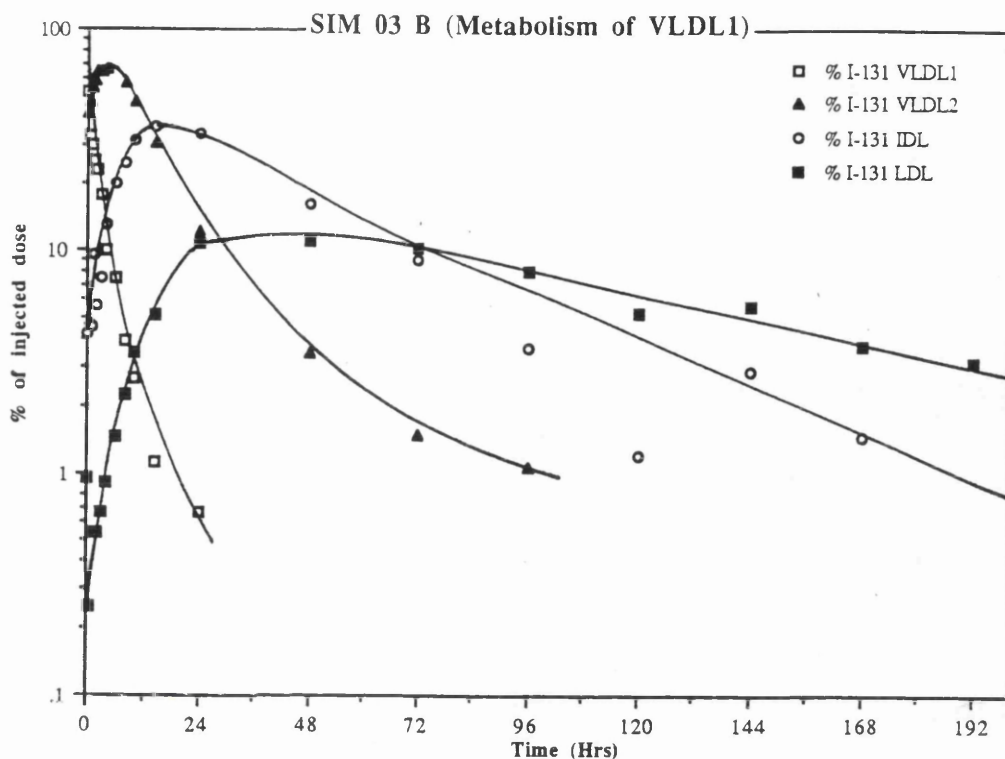
**Figure A-8.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



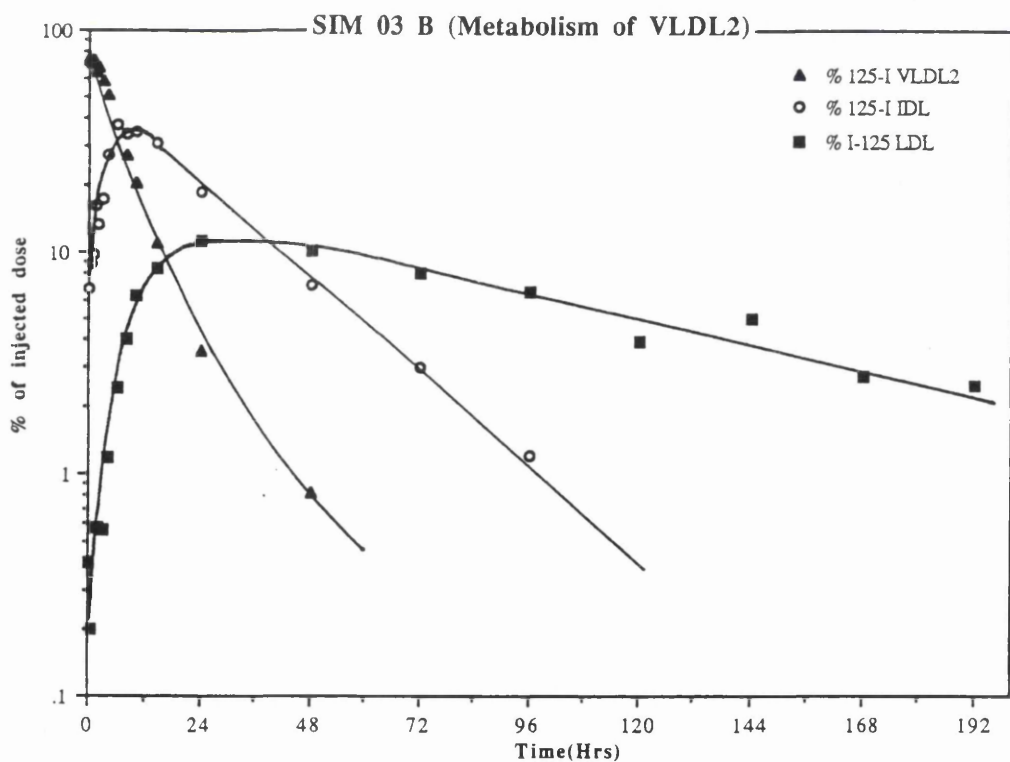
**Figure A-9.** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



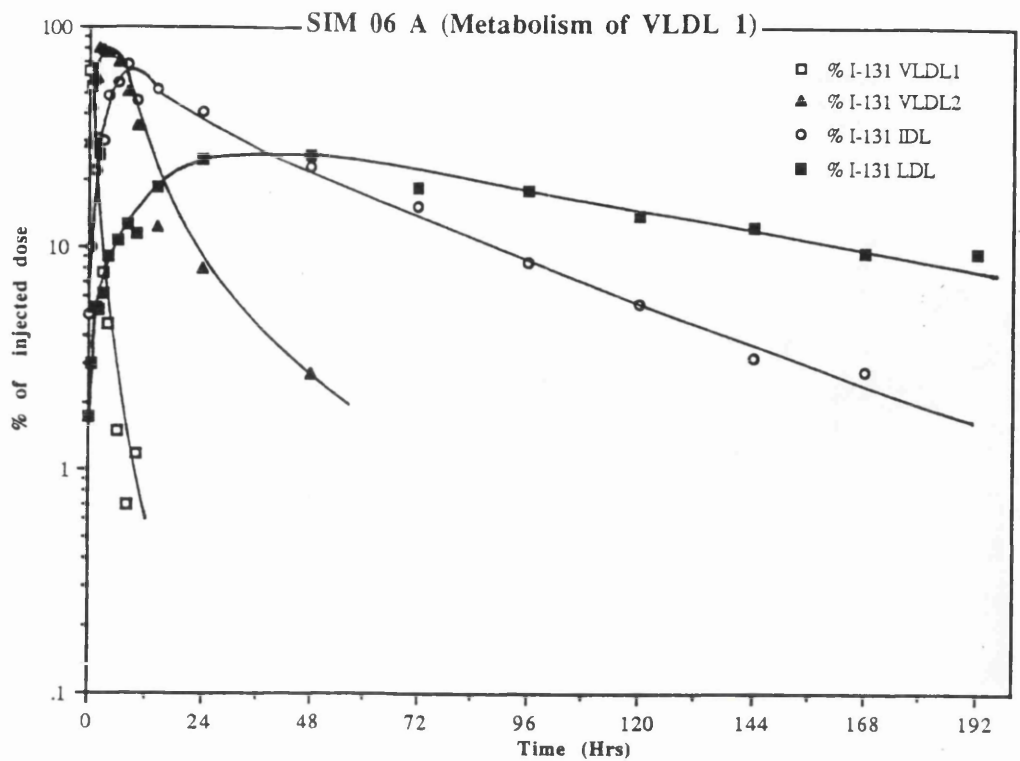
**Figure A-10** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



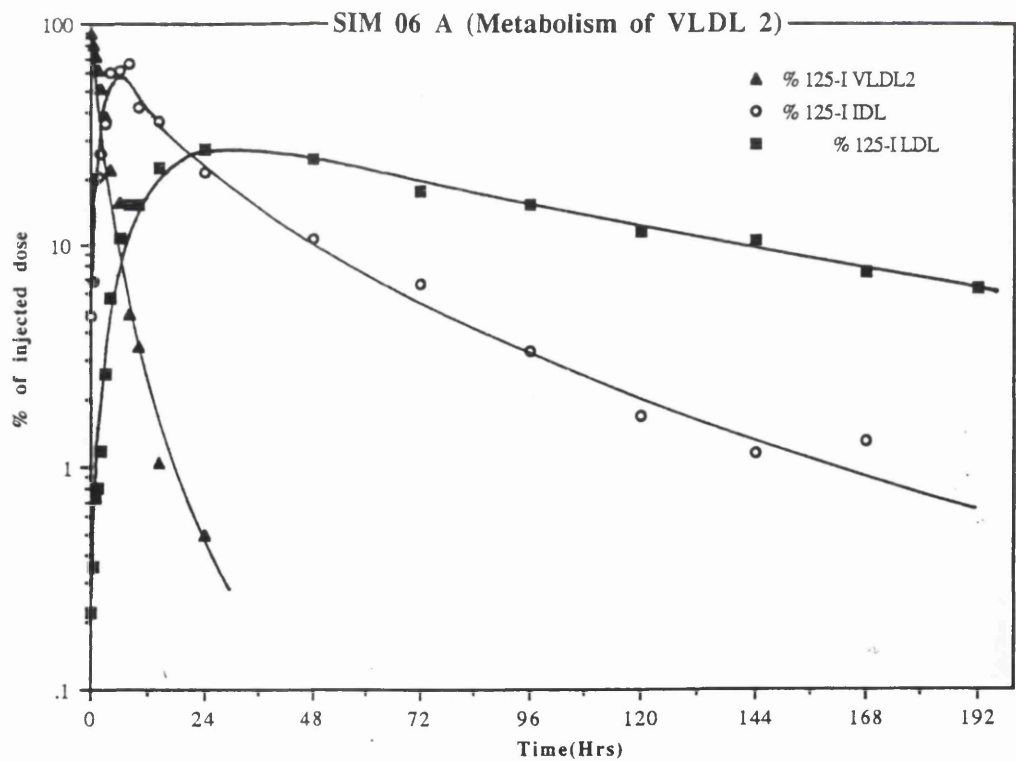
**Figure A-11** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-12** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-13** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-14** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

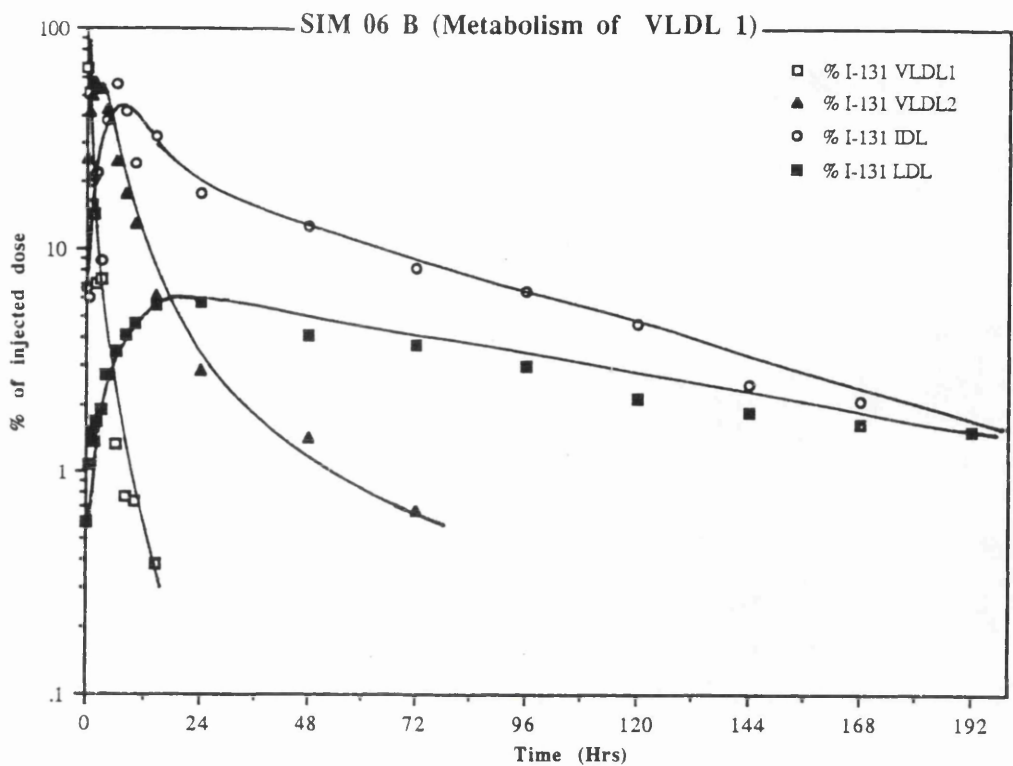


Figure A-15 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

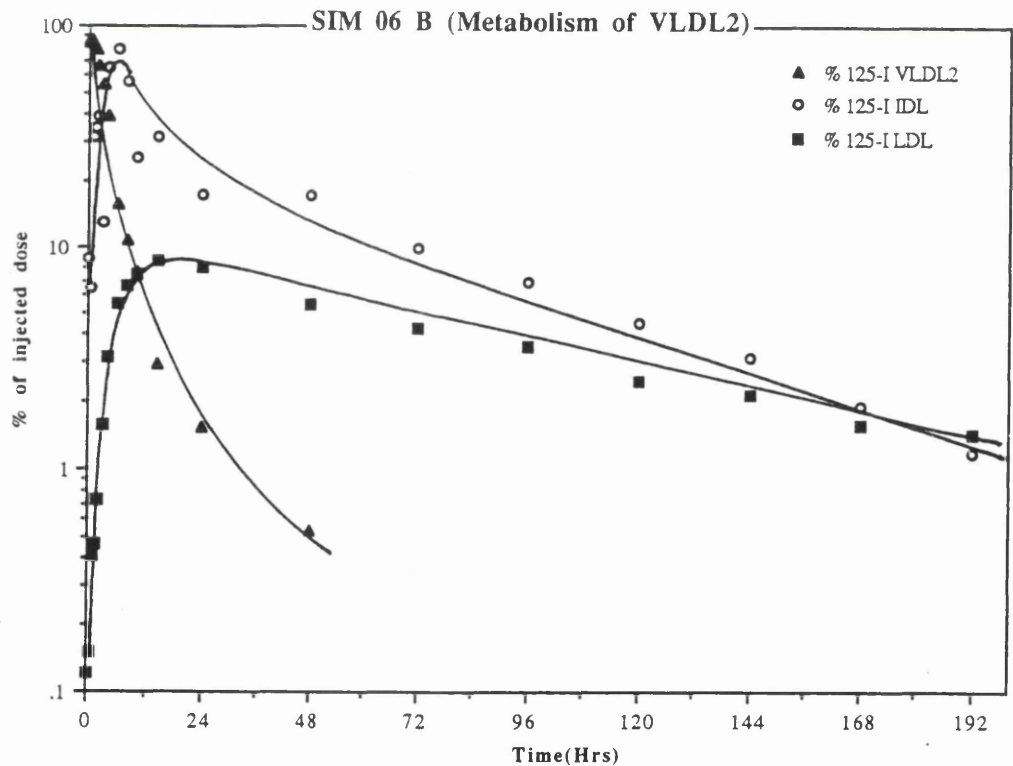
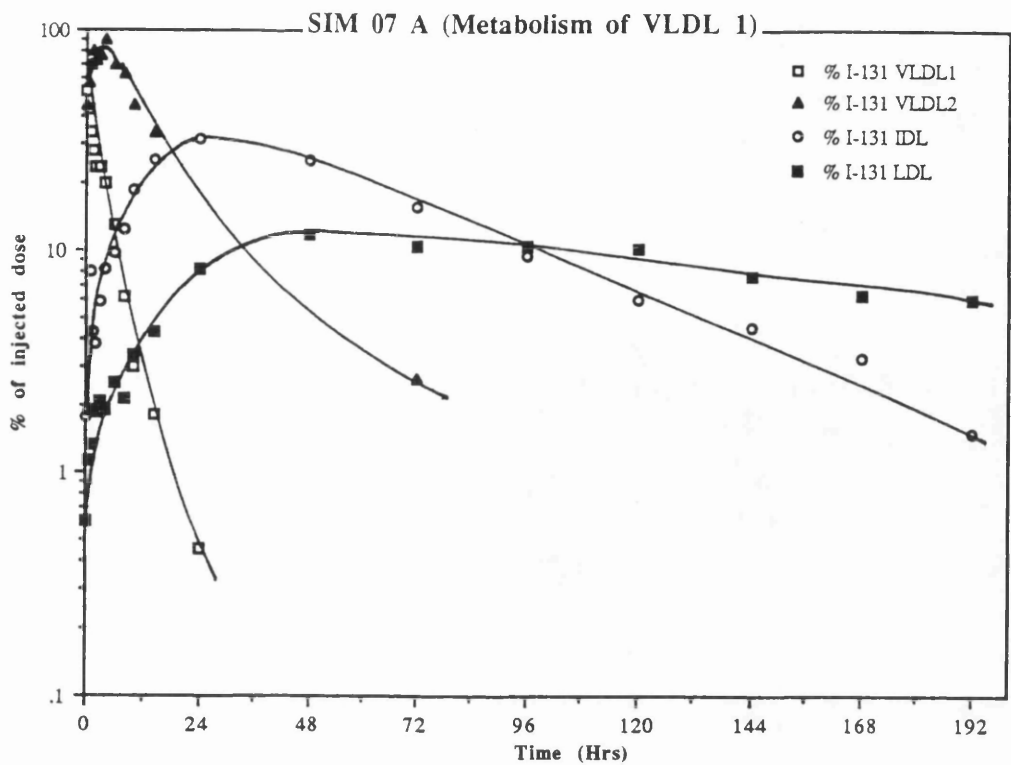
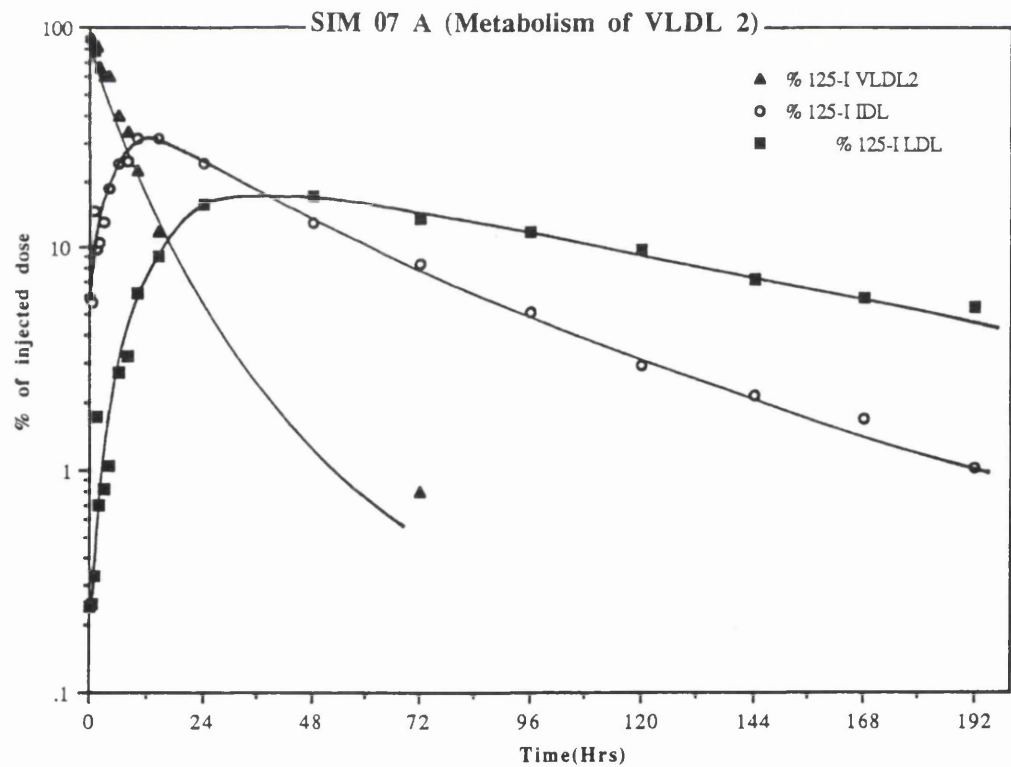


Figure A-16 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-17** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-18** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

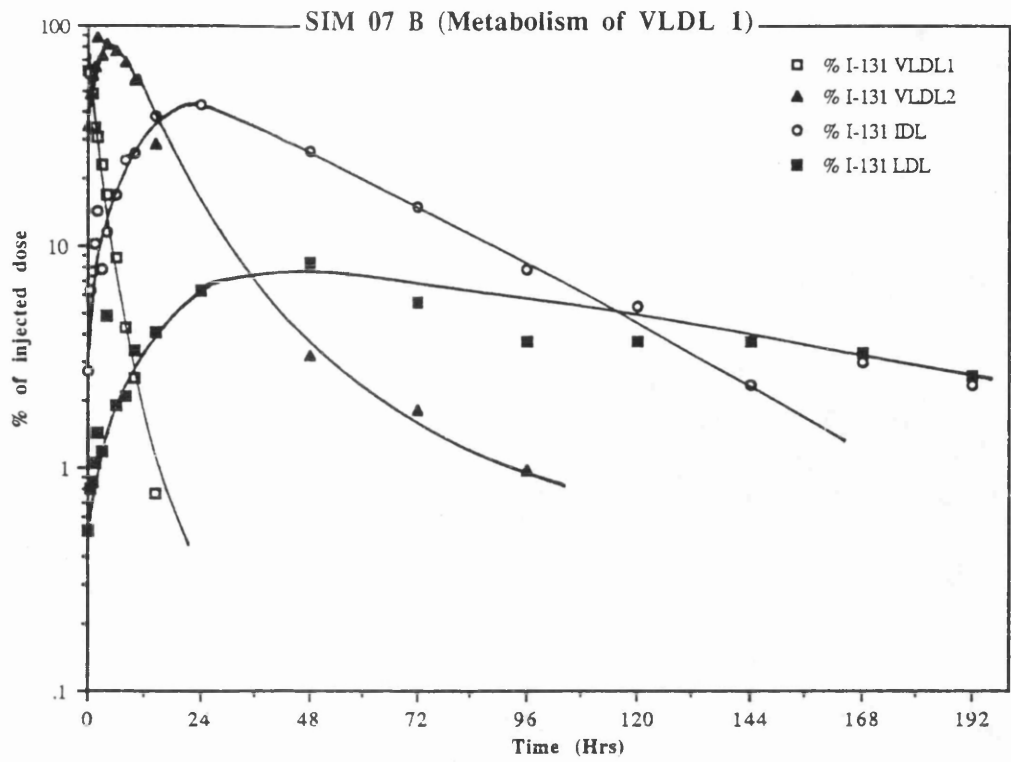


Figure A-19 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

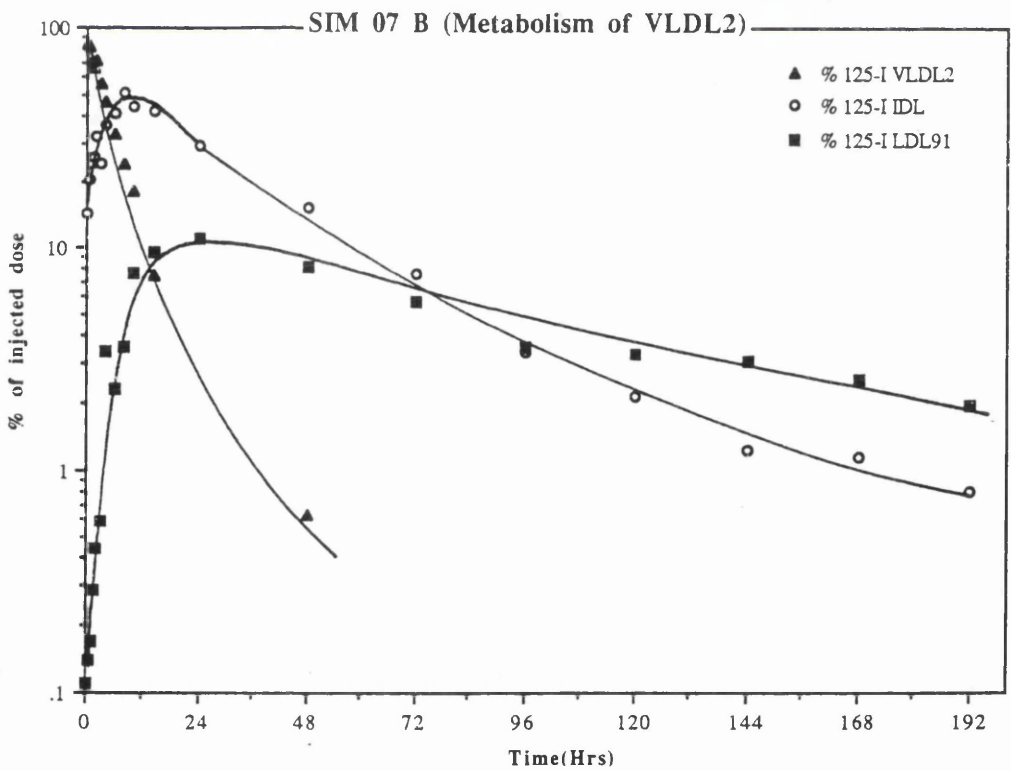
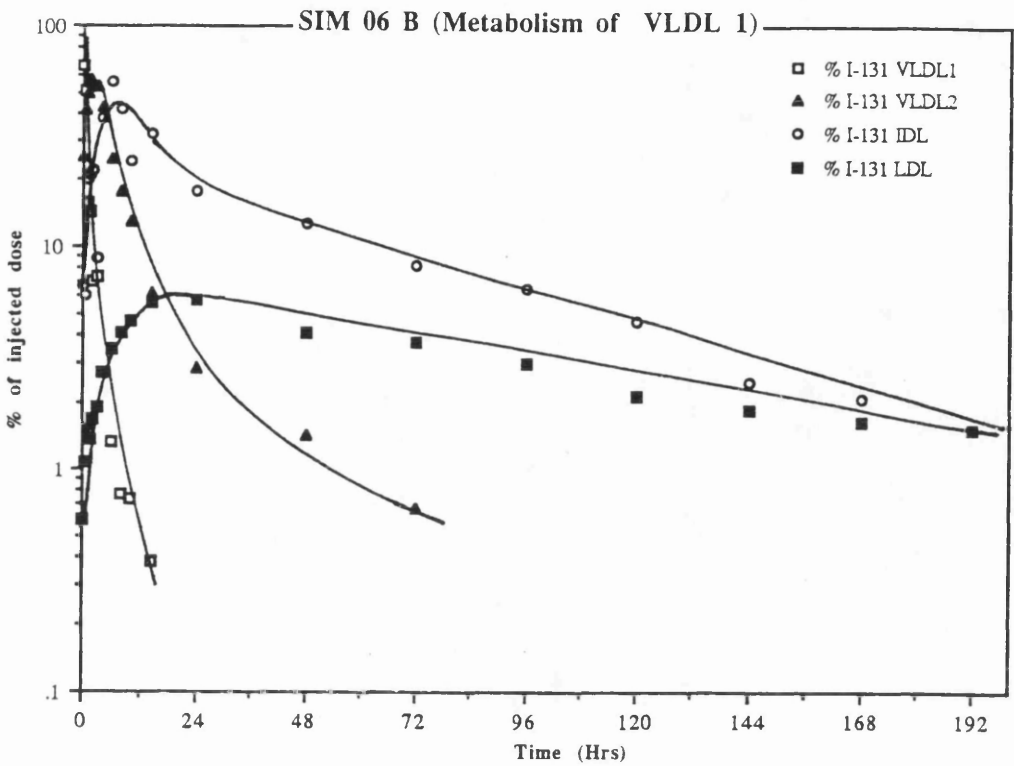
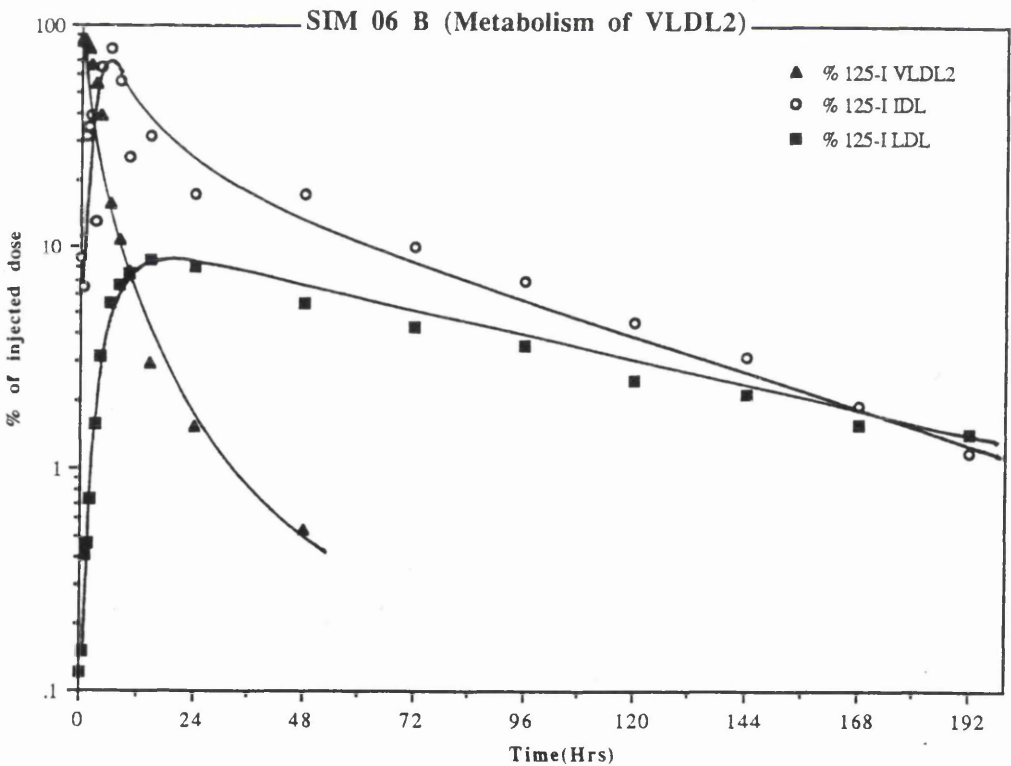


Figure A-20 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-15** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-16** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



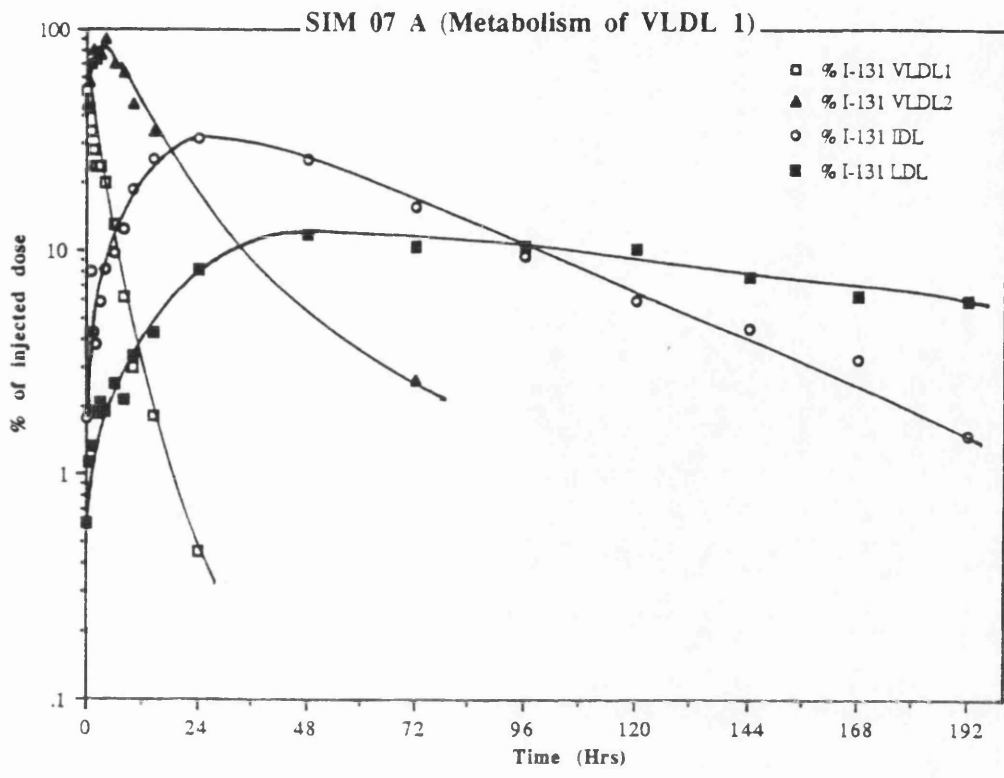


Figure A-17 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

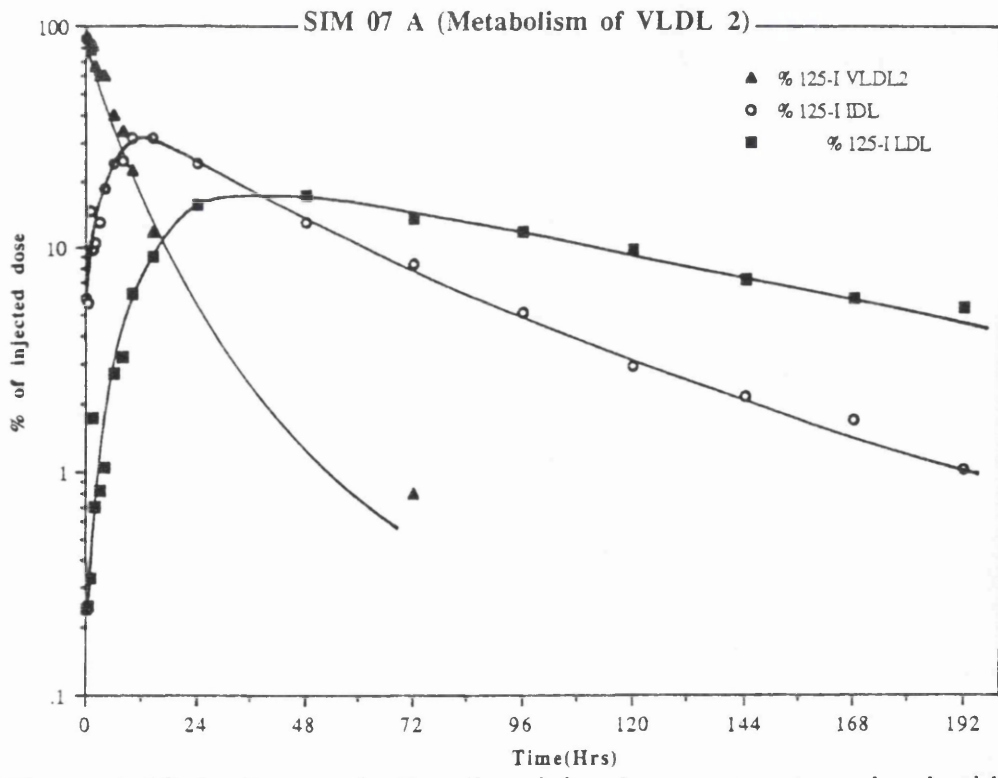


Figure A-18 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

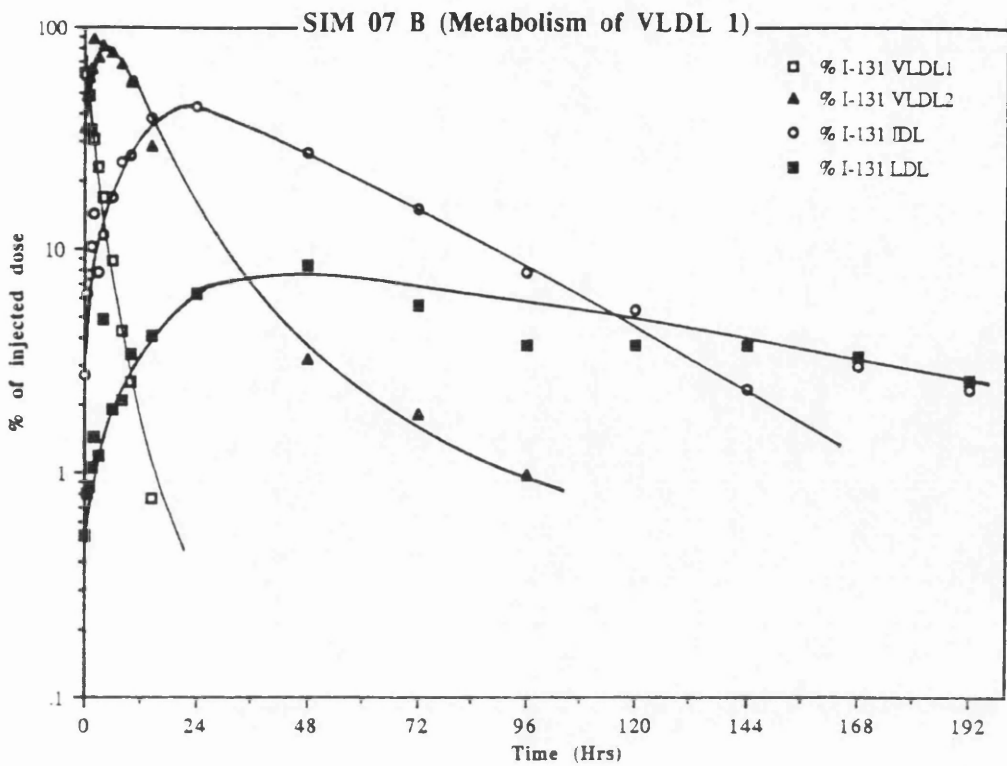


Figure A-19 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

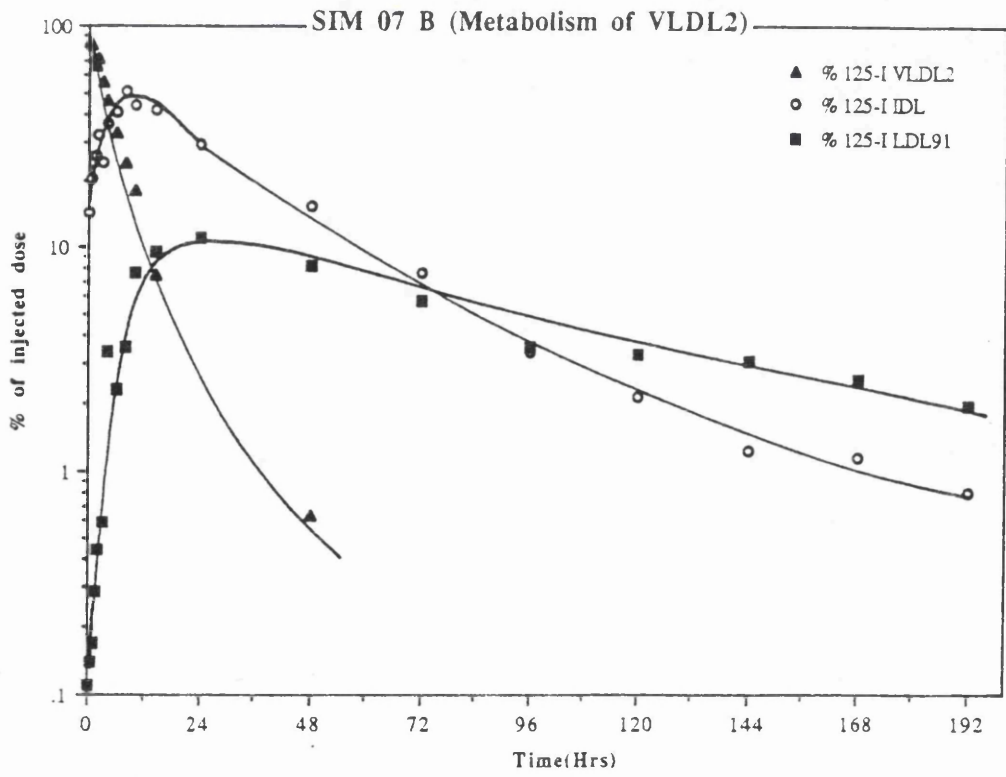
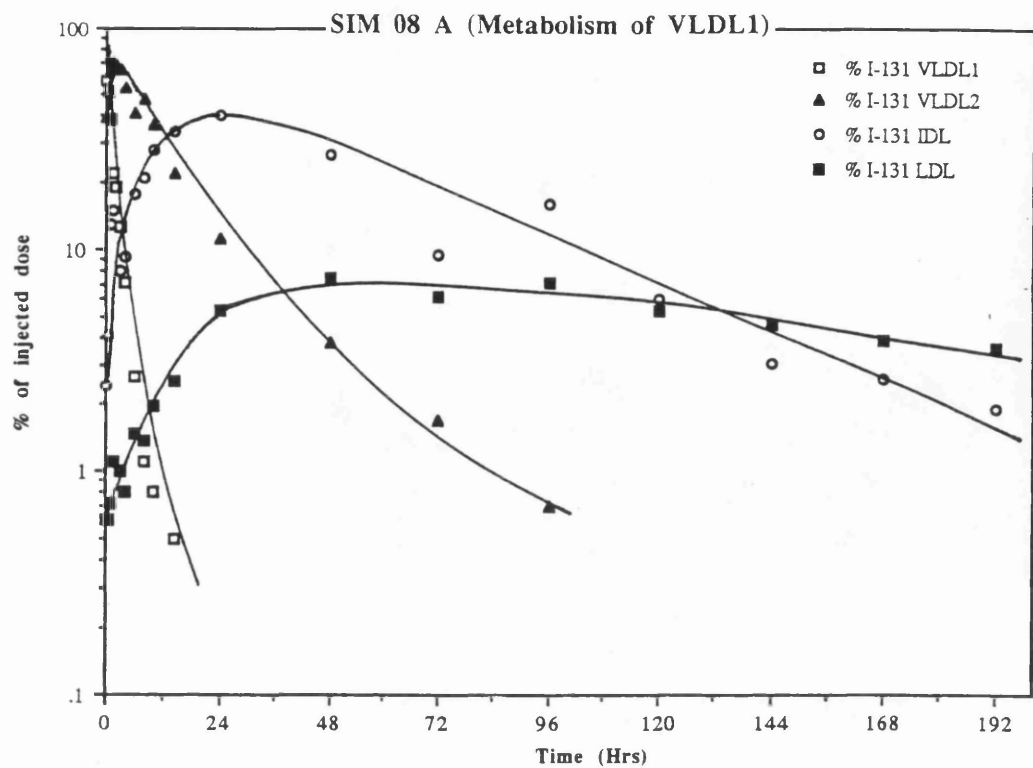
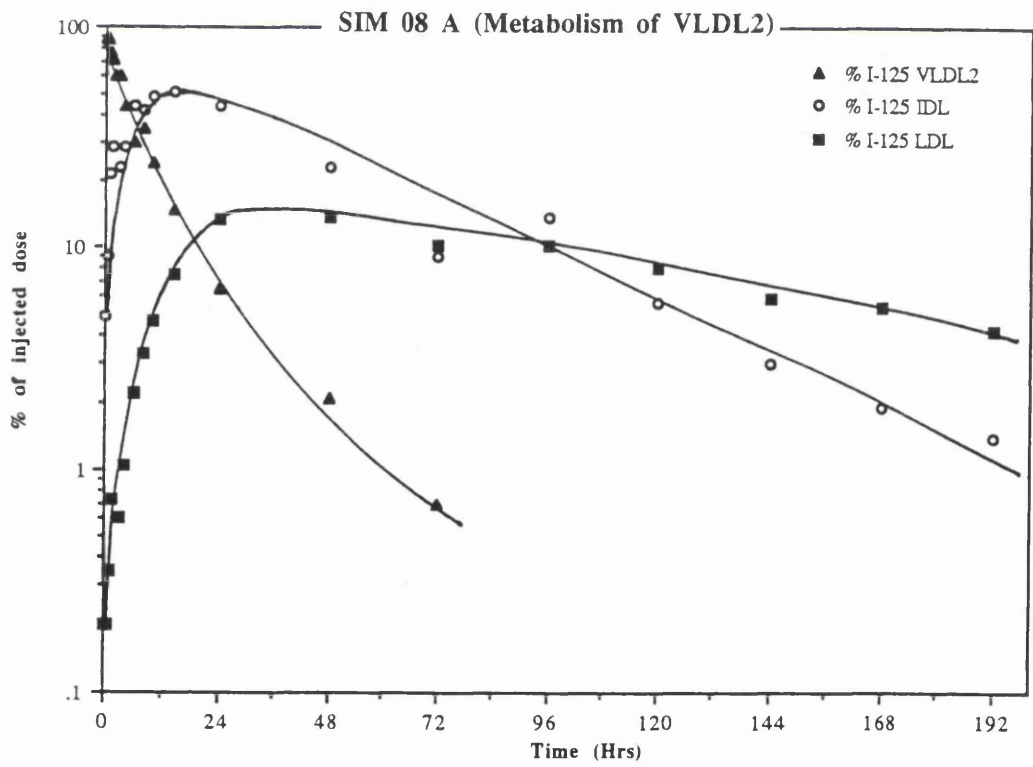


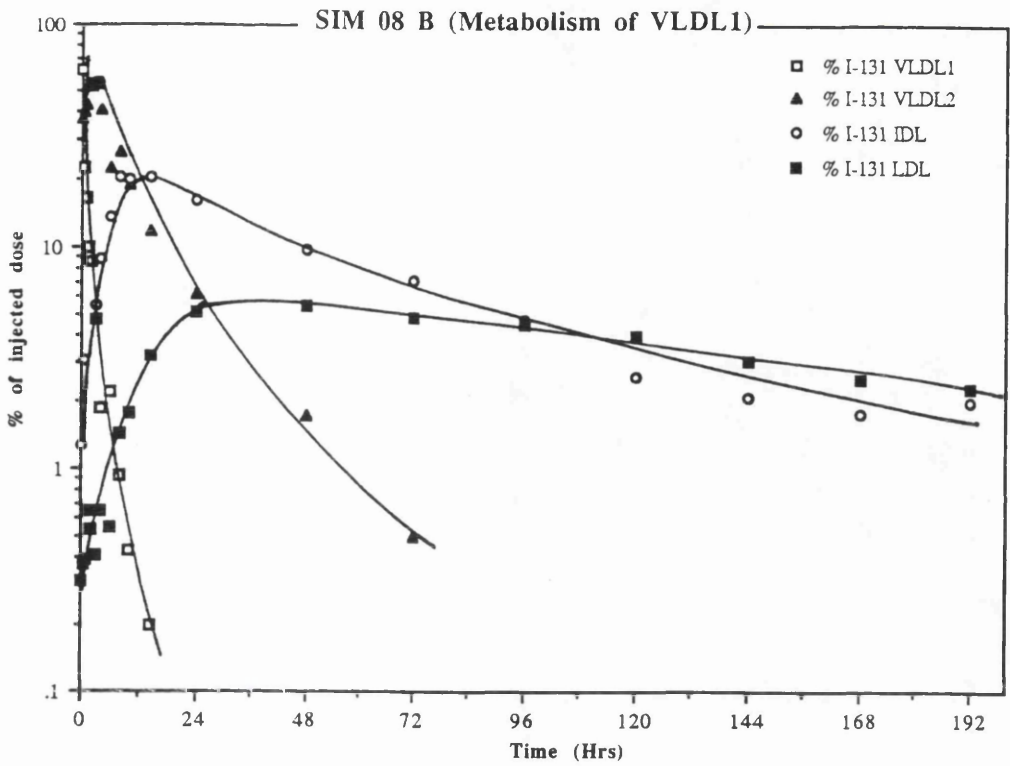
Figure A-20 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



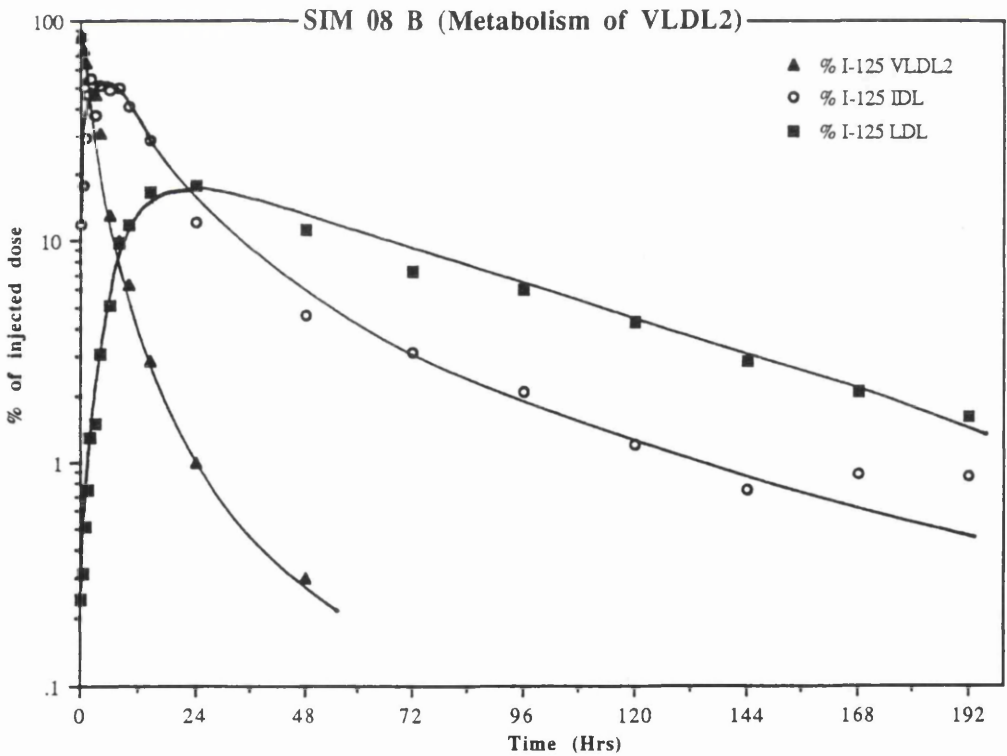
**Figure A-21** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-22** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-23** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-24** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

Table A-1. Computed Masses and Rate Constants Before and On Simvastatin Therapy. VLDL<sub>1</sub>

<i>Before Therapy</i>		L(0,1)*	L(2,1)	L(0,12)	L(1,13)	L(12,13)	M(1)†	M(12)	M(13)	U(13)‡
Subject										
SIM 01A		10.31	10.74	3.75	22.21	0.28	107	7.5	101	2280
SIM 02A		8.65	15.21	0.60	20.05	0.02	28	1.3	33	451
SIM 03A		0.97	6.50	0.40	8.51	0.02	178	9.0	157	451
SIM 05A		1.67	6.79	0.52	8.26	0.02	61	2.6	62	1333
SIM 06A		0.00	48.00	2.24	19.92	0.04	31	1.4	76	1491
SIM 07A		0.00	8.54	2.26	26.00	0.05	66	0.4	22	565
SIM 08A		17.12	18.57	1.85	15.52	0.06	20	1.4	46	716
Mean		5.53	16.34	1.66	17.21	0.07	70	3.4	71	1050
SEM		2.51	5.54	0.47	2.57	0.04	21	1.3	18	257
<i>On Therapy</i>										
SIM 01B		48.00	21.50	5.56	27.93	0.25	44	4.9	111	3085
SIM 02B		13.80	7.39	10.21	36.74	2.29	59	8.0	34	1332
SIM 03B		2.70	5.85	0.33	16.84	0.02	100	3.7	51	1332
SIM 05B		0.00	48.00	5.91	12.83	0.13	16	1.3	59	776
SIM 06B		24.00	48.00	3.47	32.38	0.18	48	5.0	107	3473
SIM 07B		0.00	15.70	1.26	9.73	0.02	29	0.9	47	456
SIM 08B		10.50	10.41	4.60	29.54	0.83	23	3.0	17	495
Mean		14.14	22.41	4.48	23.71	0.53	46	3.8	61	1196
SEM		6.52	6.90	1.24	3.96	0.31	11	0.9	13	475

\*Rate constants, L(destination, source) are in units, d<sup>-1</sup> † Masses M( ) are in mg. ‡ U( ) represents *de novo* synthesis of apo B into a compartment

Table A-2. Computed Masses and Rate Constants Before and On Simvastatin Therapy. VLDL<sub>2</sub>

<i>Before Therapy</i>										
Subject	L(4,2)	L(6,2)	L(0,6)	L(0,4)	L(8,4)	L(11,4)	L(9,4)	L(0,5)	L(7,5)	L(10,5)
SIM 01A	5.80	0.95	3.67	16.25	9.61	0.00	1.27	7.46	2.43	0.00
SIM 02A	7.57	0.06	0.57	2.04	2.08	0.00	1.68	3.36	2.50	0.00
SIM 03A	24.00	0.75	0.37	0.36	0.75	0.00	0.24	1.15	2.90	0.00
SIM 05A	3.90	0.10	0.72	0.56	1.78	0.00	4.05	1.48	2.11	0.00
SIM 06A	24.00	0.04	0.34	0.00	3.70	0.02	1.23	0.00	8.18	0.00
SIM 07A	8.04	0.07	0.39	1.03	0.38	0.00	1.13	2.55	2.09	0.00
SIM 08A	15.95	0.18	0.47	0.00	1.81	0.00	0.48	2.49	5.44	0.00
Mean	12.75	0.31	0.93	2.89	2.87	0.00	1.44	2.64	3.66	0.00
SEM	3.23	0.14	0.46	2.24	1.19	0.00	0.47	0.90	0.87	0.00
<i>On Therapy</i>										
SIM 01B	12.12	2.63	4.38	5.54	8.75	0.08	0.35	12.65	2.21	0.12
SIM 02B	8.26	0.05	1.33	3.32	3.93	0.32	0.38	4.40	6.80	0.00
SIM 03B	6.88	0.05	0.39	0.00	2.06	0.00	1.27	1.78	2.12	0.00
SIM 05B	3.70	0.00	0.32	0.00	2.41	0.00	1.22	0.84	3.97	0.00
SIM 06B	5.55	0.01	0.29	24.00	4.57	2.40	0.00	3.60	2.40	0.00
SIM 07B	9.72	0.08	0.50	0.35	2.39	0.00	0.86	1.57	3.20	0.00
SIM 08B	6.38	0.17	0.87	2.19	3.10	0.00	1.02	2.95	3.81	0.00
Mean	7.52	0.43	1.15	5.06	3.89	0.40	0.73	3.97	3.50	0.02
SEM	1.06	0.37	0.56	3.25	0.88	0.34	0.19	1.52	0.62	0.02

**Table A-3.**Computed Masses and Rate Constants Before and On Simvastatin Therapy. VLDL<sub>2</sub> (contd)

<i>Before Therapy</i>		M(2)	M(4)	M(6)	M(5)	U(5)
Subject						
SIM 01A		169	36	44	310	3065
SIM 02A		55	72	5	172	1008
SIM 03A		47	829	95	382	1547
SIM 05A		103	63	14	287	1030
SIM 06A		63	305	7	19	155
SIM 07A		70	225	12	210	974
SIM 08A		23	161	9	104	825
Mean		76	242	27	212	1229
SEM		18	105	12	48	343
<i>On Therapy</i>						
SIM 01B		65	53	39	273	4088
SIM 02B		52	54	2	256	2867
SIM 03B		84	174	10	450	1754
SIM 05B		205	209	0	124	596
SIM 06B		415	74	10	113	678
SIM 07B		47	127	8	237	1132
SIM 08B		37	37	7	294	1990
Mean		129	104	11	250	1872
SEM		52	25	5	43	476

Table A-4. Computed Masses and Rate Constants Before and On Simvastatin Therapy. IDL

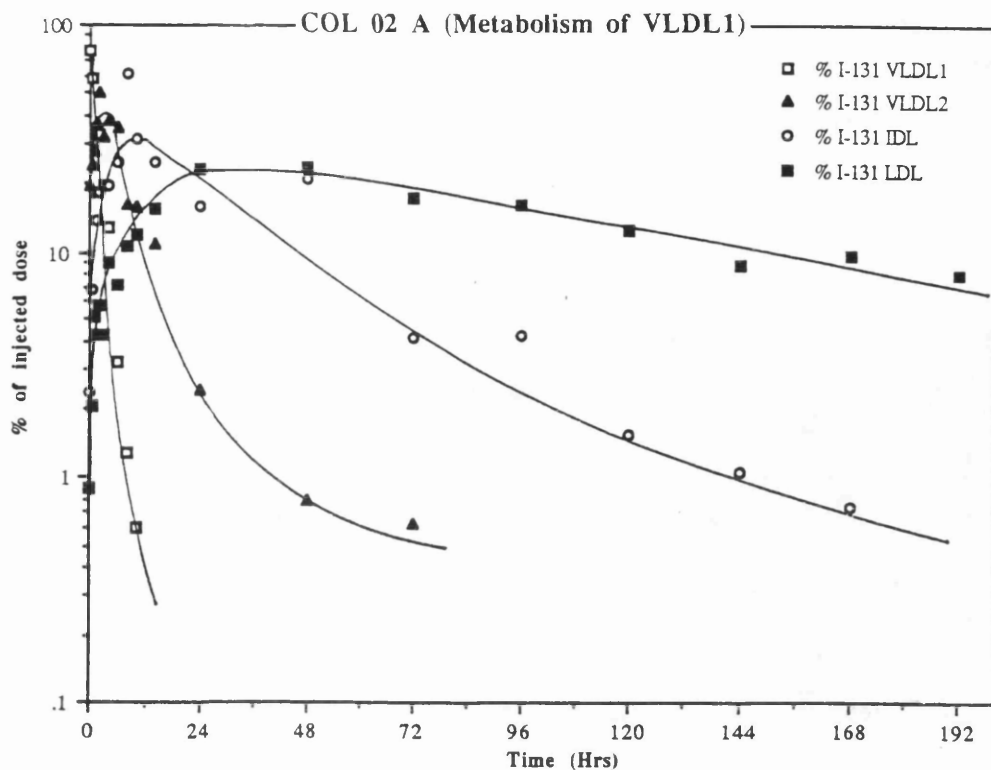
<i>Before Therapy</i>		<i>On Therapy</i>							
Subject	L(0,8)	L(11,8)	L(0,9)	L(0,7)	L(10,7)	M(8)	M(9)	M(7)	
SIM 01A	0.99	1.28	0.29	0.00	1.85	153	158	404	
SIM 02A	1.36	0.96	0.36	0.35	1.18	65	332	281	
SIM 03A	0.40	0.30	13.73	0.66	0.90	887	14	707	
SIM 05A	0.00	1.50	0.49	0.85	0.70	75	518	390	
SIM 06A	1.73	1.22	0.47	0.00	2.48	383	803	64	
SIM 07A	0.00	1.91	0.42	0.34	0.72	41	605	412	
SIM 08A	0.88	0.26	0.25	0.82	0.44	255	307	449	
Mean	0.77	1.06	2.29	0.43	1.18	266	391	387	
SEM	0.25	0.23	1.91	0.14	0.28	113	102	73	
SIM 01B	2.06	0.63	0.20	1.10	1.31	173	93	249	
SIM 02B	1.18	1.26	0.16	5.02	3.35	87	131	208	
SIM 03B	1.18	0.65	0.66	1.59	0.46	195	334	456	
SIM 05B	5.48	0.92	0.64	0.91	0.87	80	400	275	
SIM 06B	0.41	0.04	0.08	0.72	0.17	757	0	305	
SIM 07B	2.56	0.37	0.35	1.37	0.24	103	311	472	
SIM 08B	0.71	0.27	0.30	1.93	0.58	119	129	447	
Mean	1.94	0.59	0.34	1.81	1.00	216	200	345	
SEM	0.65	0.16	0.09	0.56	0.42	92	56	42	



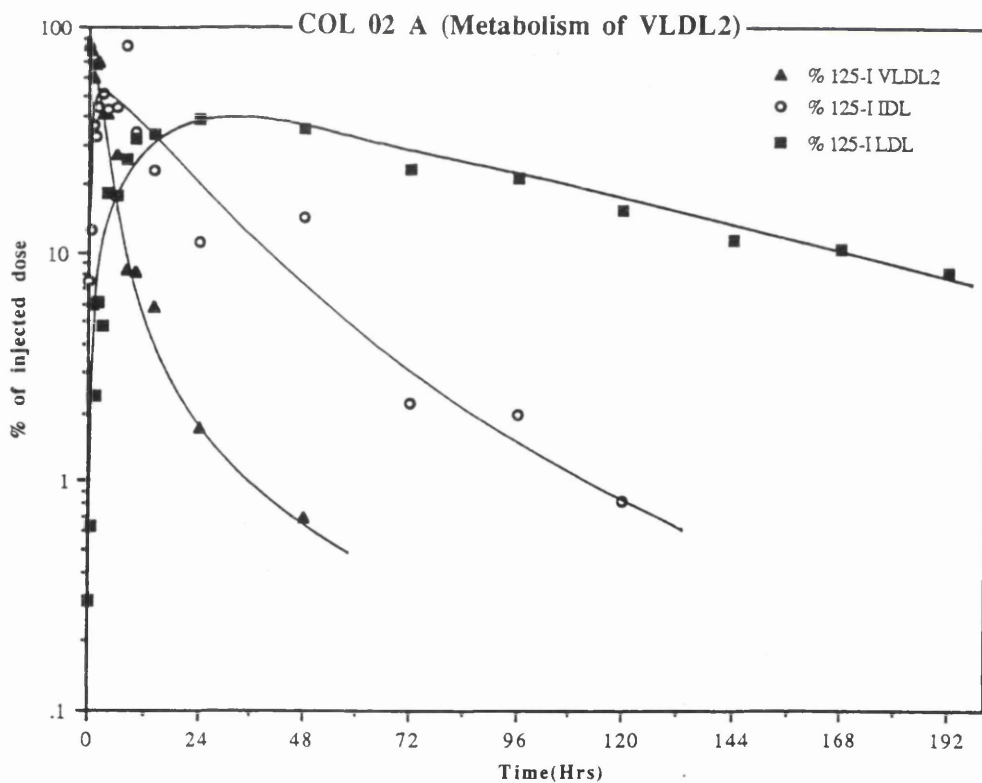
Table A-5. Computed Masses and Rate Constants Before and On Simvastatin Therapy. LDL

<i>Before Therapy</i>					
Subject	L(0,11)	L(0,10)	M(11)	M(10)	
SIM 01A	0.16	0.46	1256	1654	
SIM 02A	0.17	0.40	354	839	
SIM 03A	0.35	0.23	767	2825	
SIM 05A	0.13	0.21	840	1320	
SIM 06A	0.20	0.35	2412	454	
SIM 07A	0.13	0.23	627	1291	
SIM 08A	0.18	0.19	374	1044	
Mean	0.19	0.30	949	1347	
SEM	0.03	0.04	270	286	
<i>On Therapy</i>					
SIM 01B	0.28	0.25	416	1421	
SIM 02B	0.30	0.80	430	886	
SIM 03B	0.23	0.25	549	857	
SIM 05B	0.22	0.31	336	778	
SIM 06B	0.48	0.24	871	107	
SIM 07B	0.17	0.22	220	513	
SIM 08B	0.21	0.36	153	715	
Mean	0.27	0.35	425	751	
SEM	0.04	0.08	90	150	

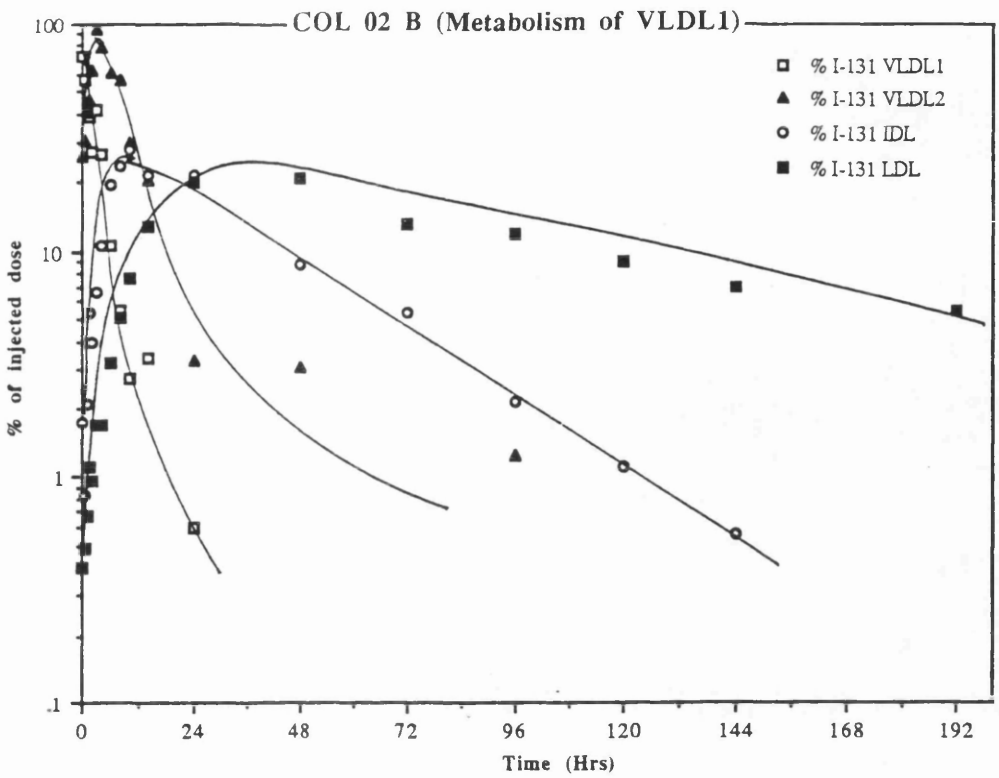
*Appendix 3*



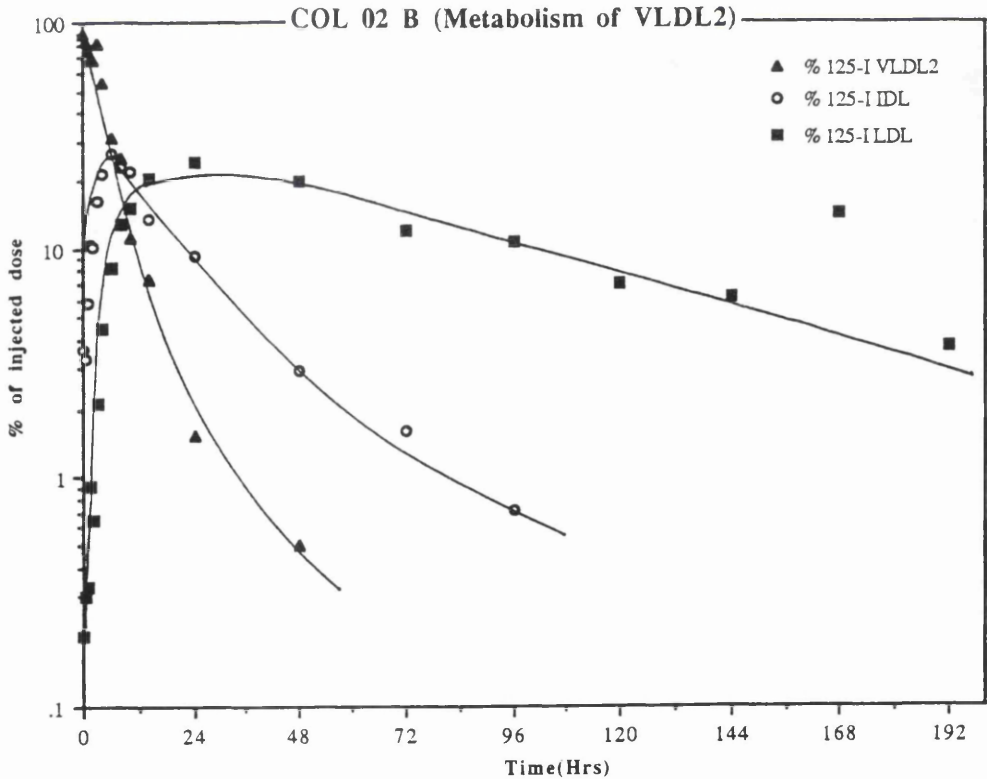
**Figure A-25** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



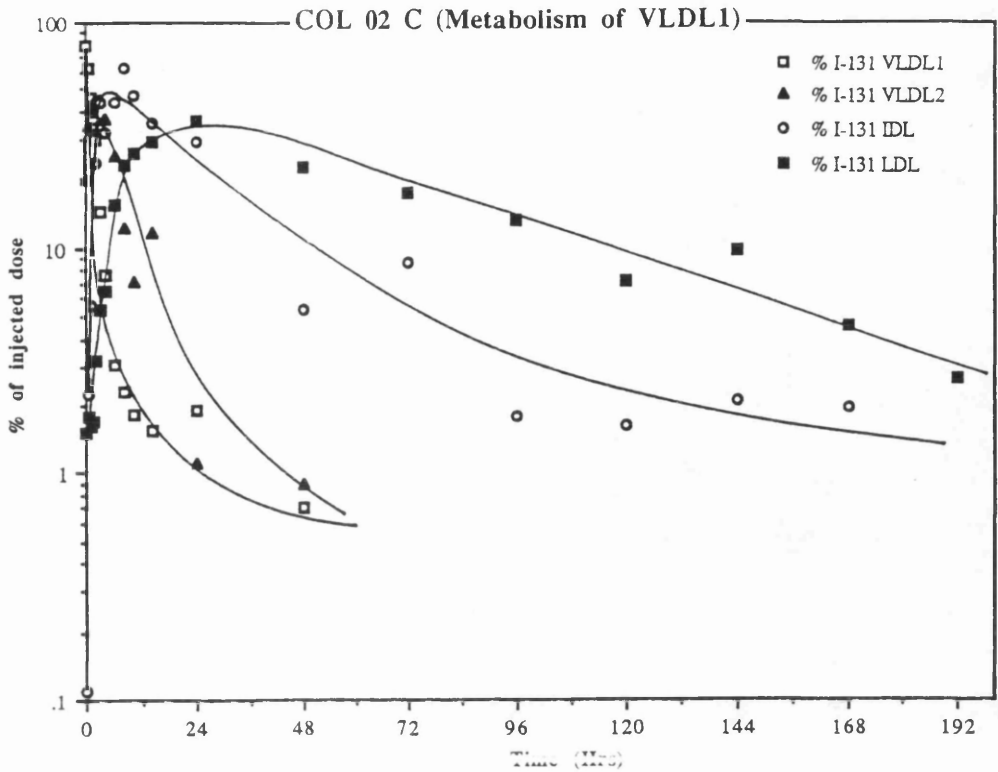
**Figure A-26** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



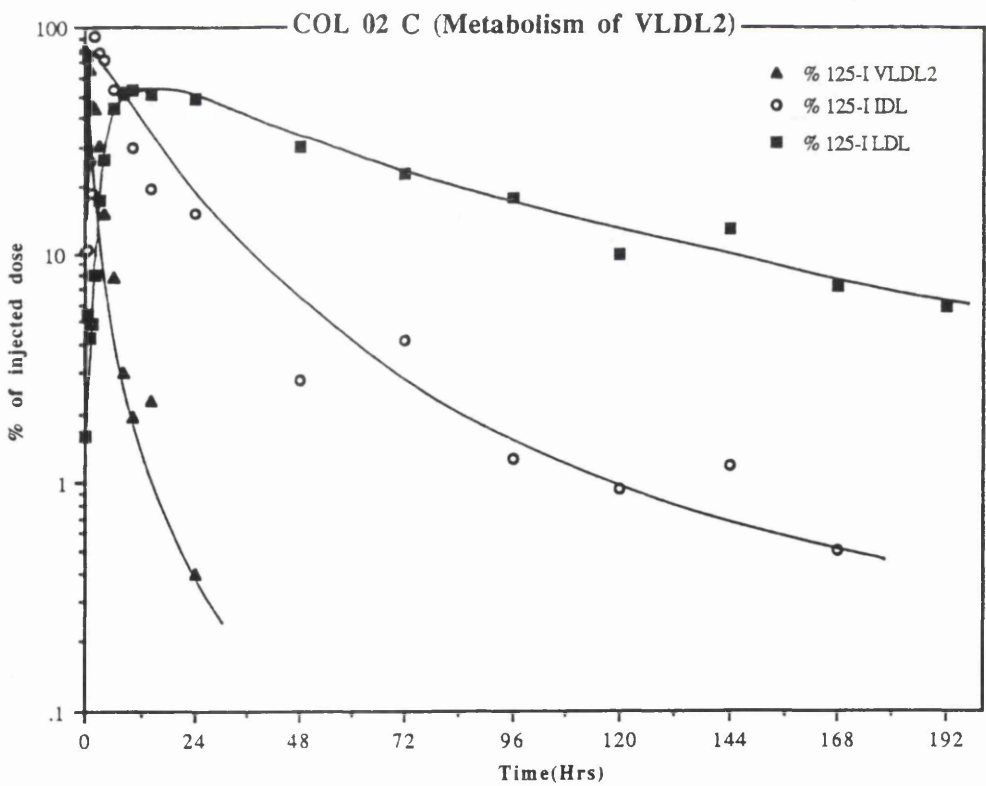
**Figure A-27** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



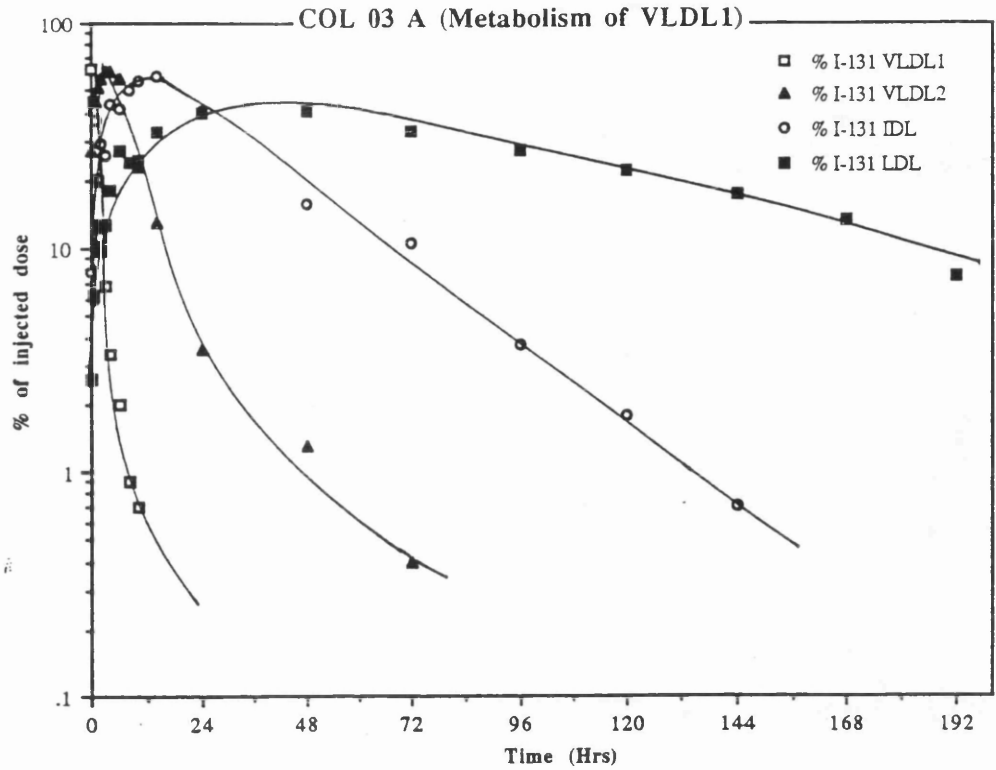
**Figure A-28** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



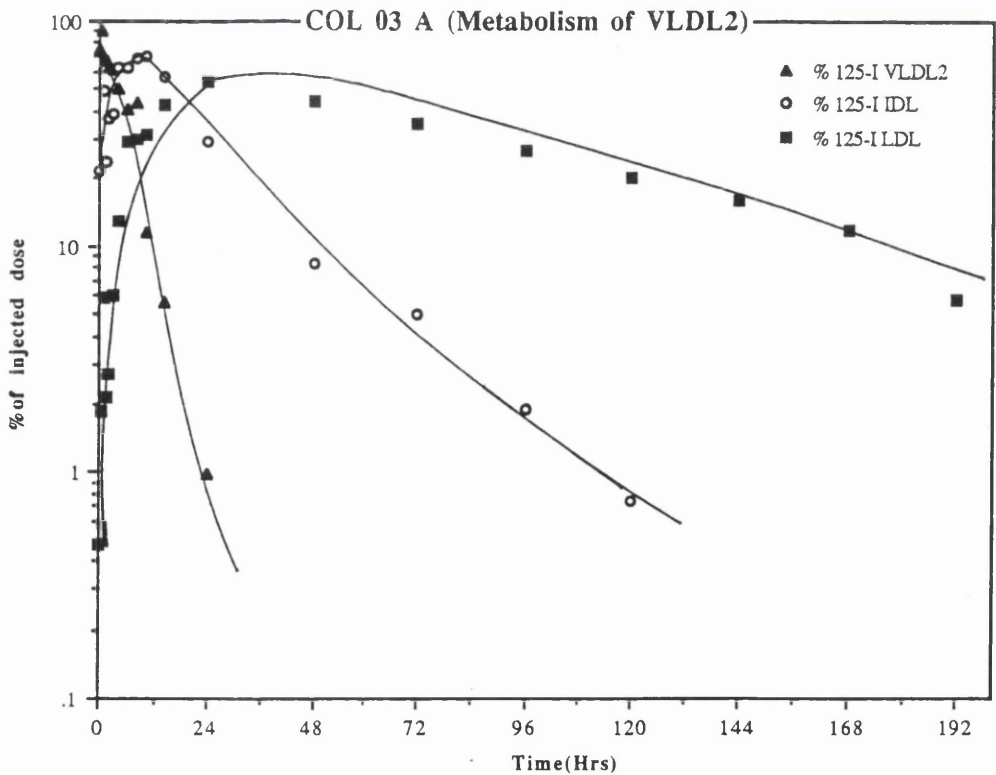
**Figure A-29** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



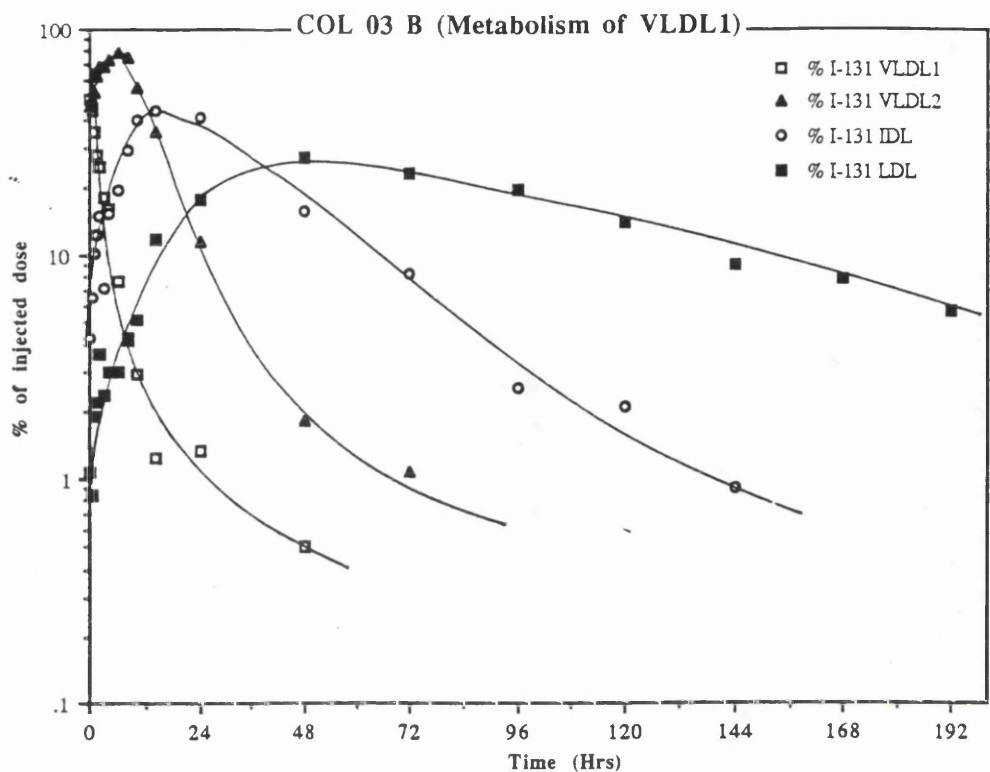
**Figure A-30** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



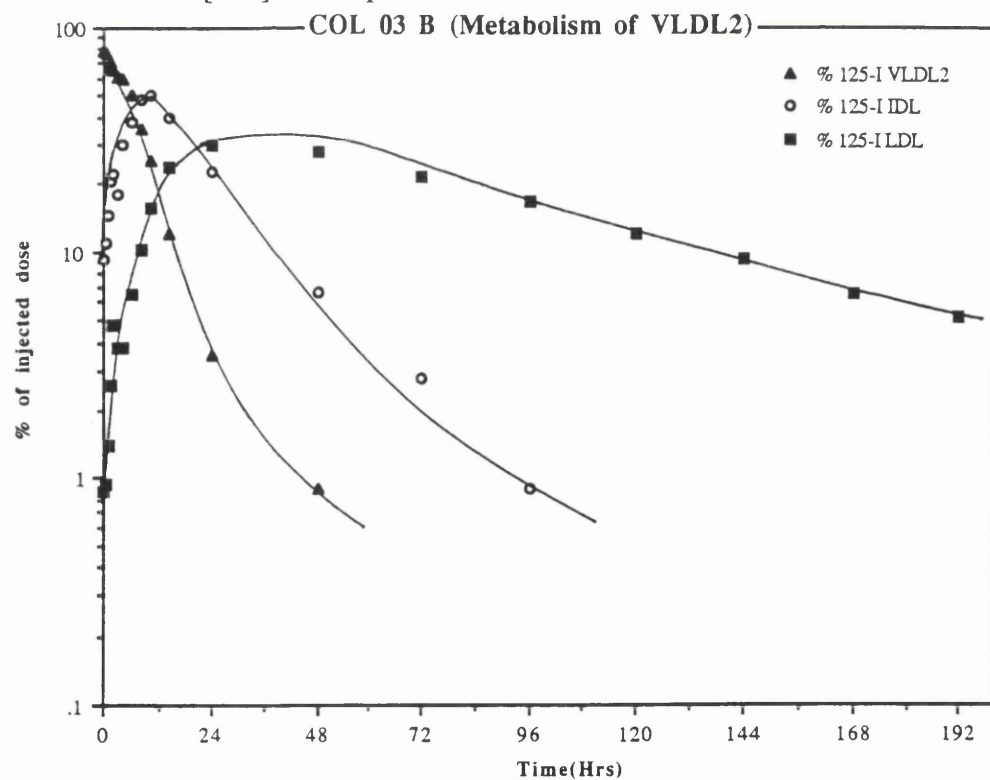
**Figure A-31** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



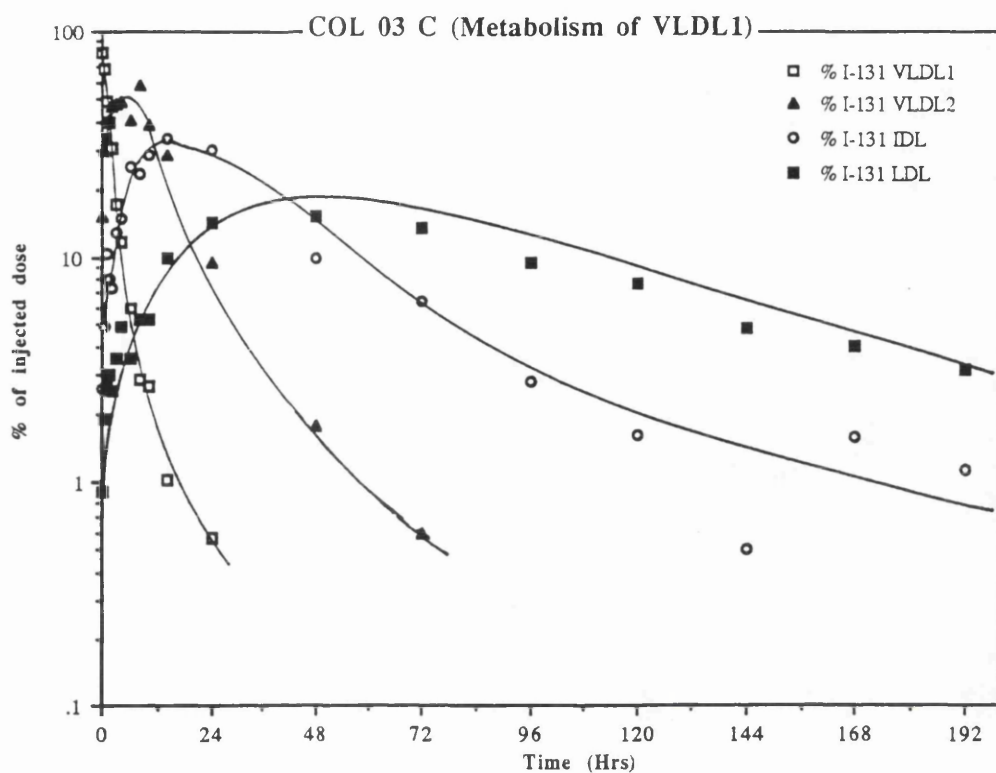
**Figure A-32** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



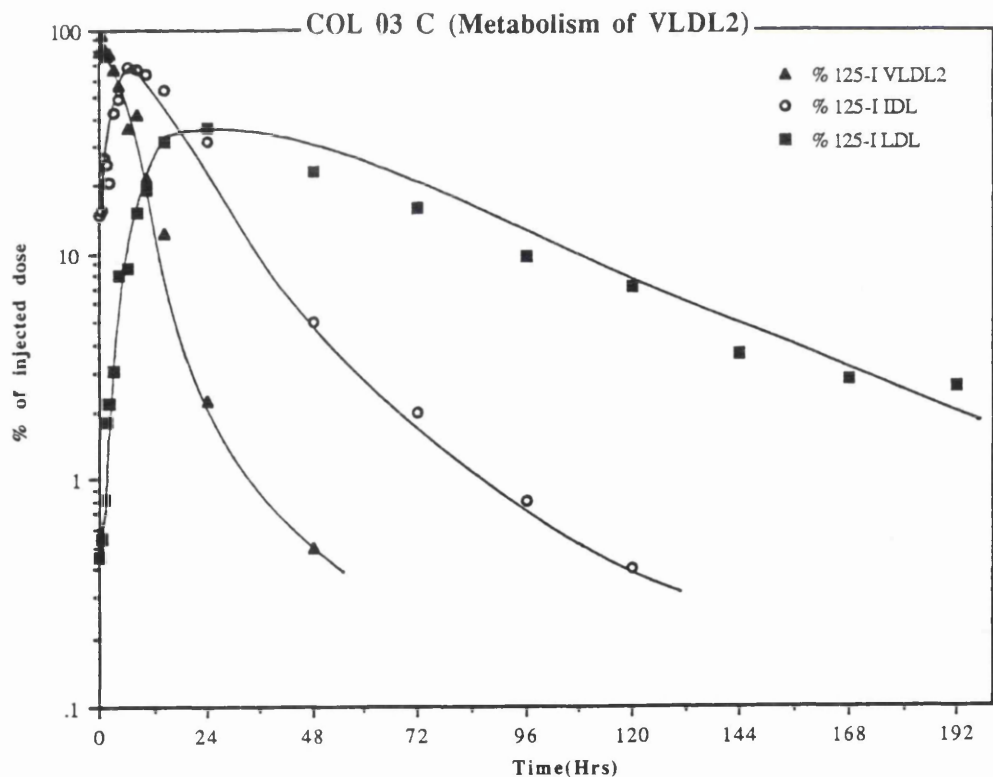
**Figure A-33** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-34** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

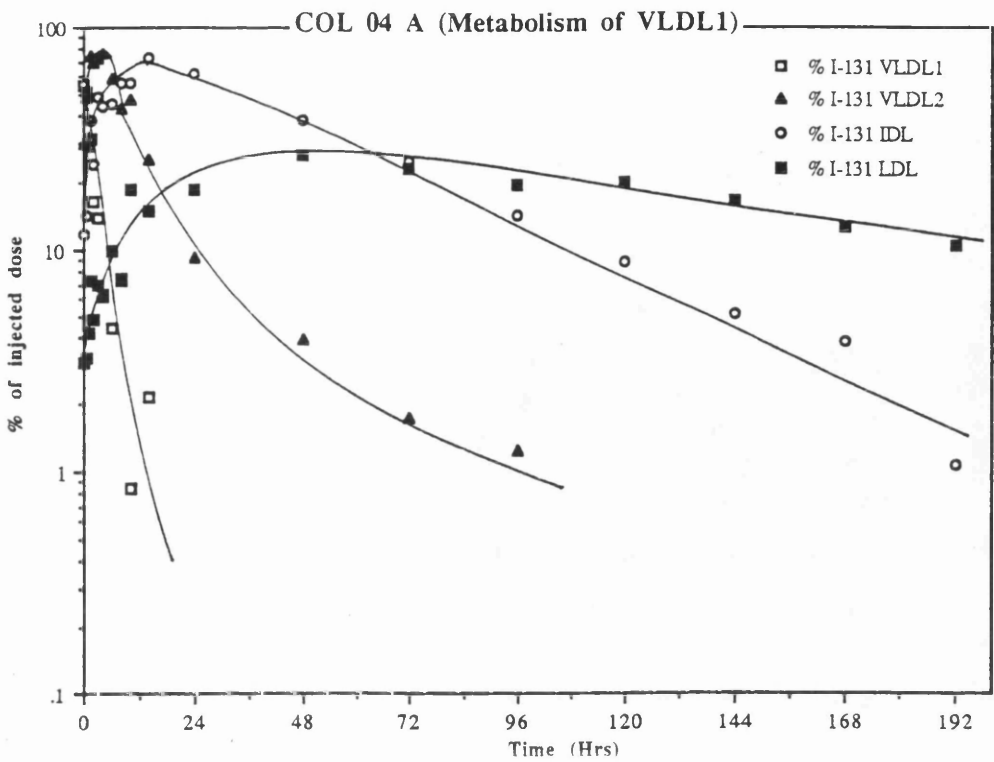


**Figure A-35** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

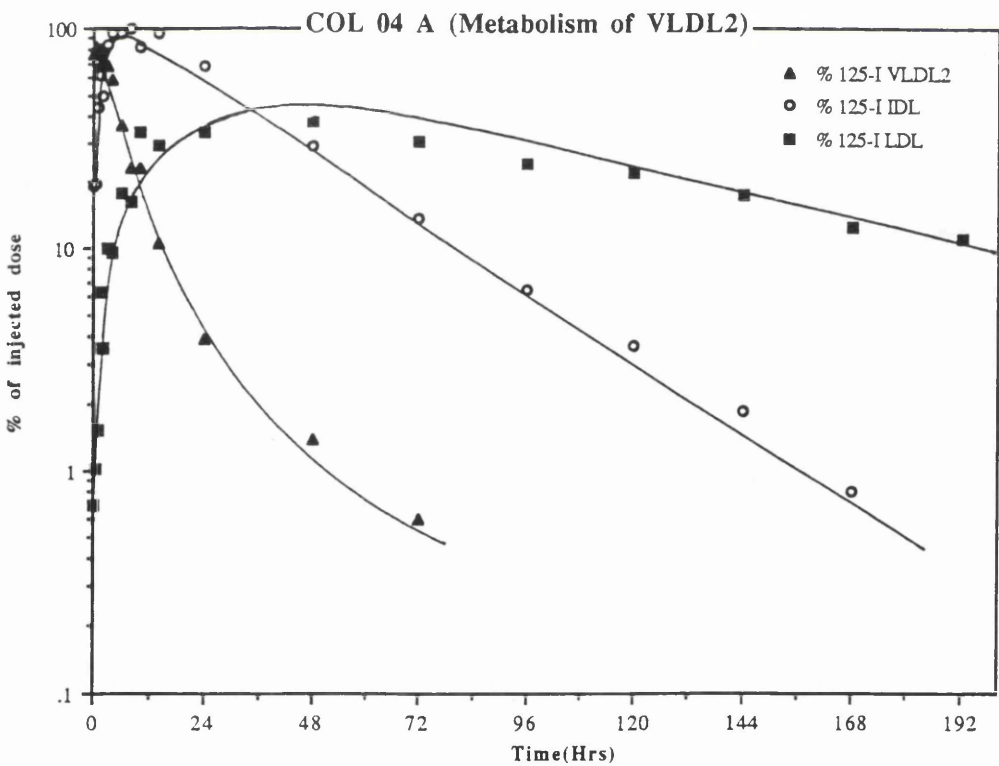


**Figure A-36** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

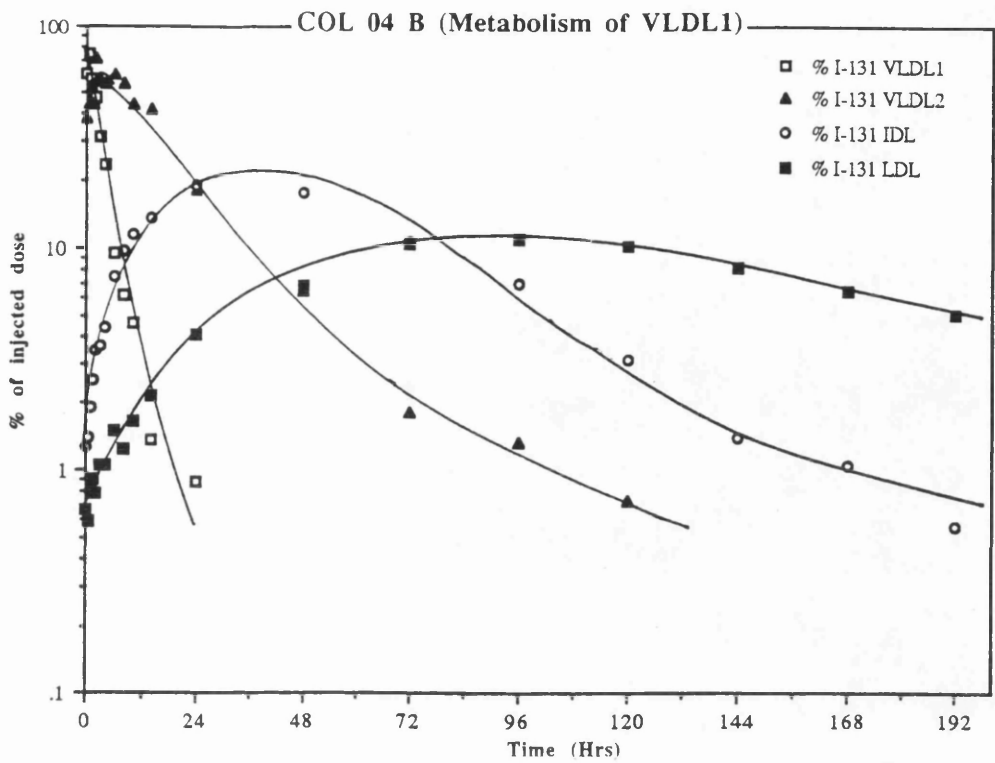




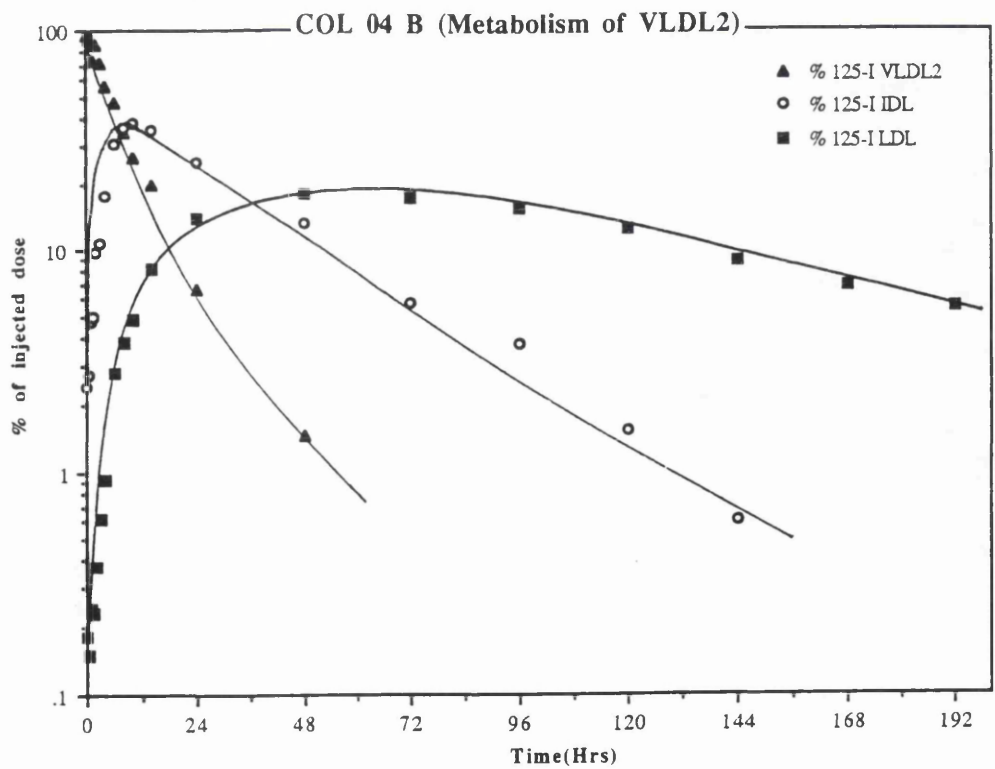
**Figure A-37** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



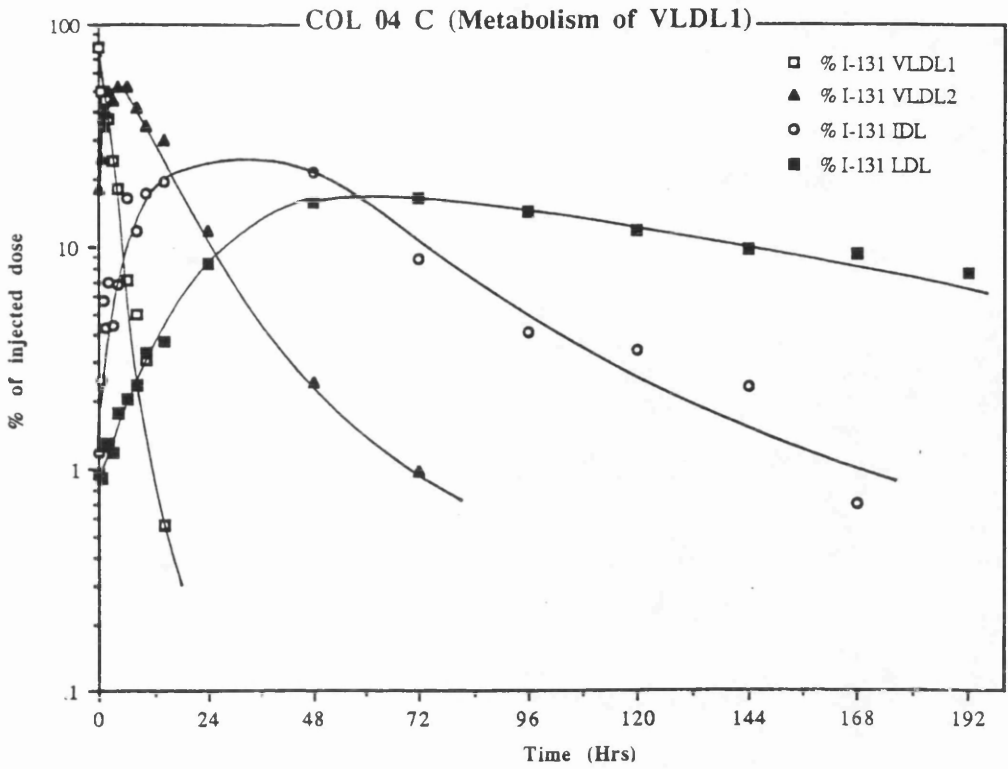
**Figure A-38** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



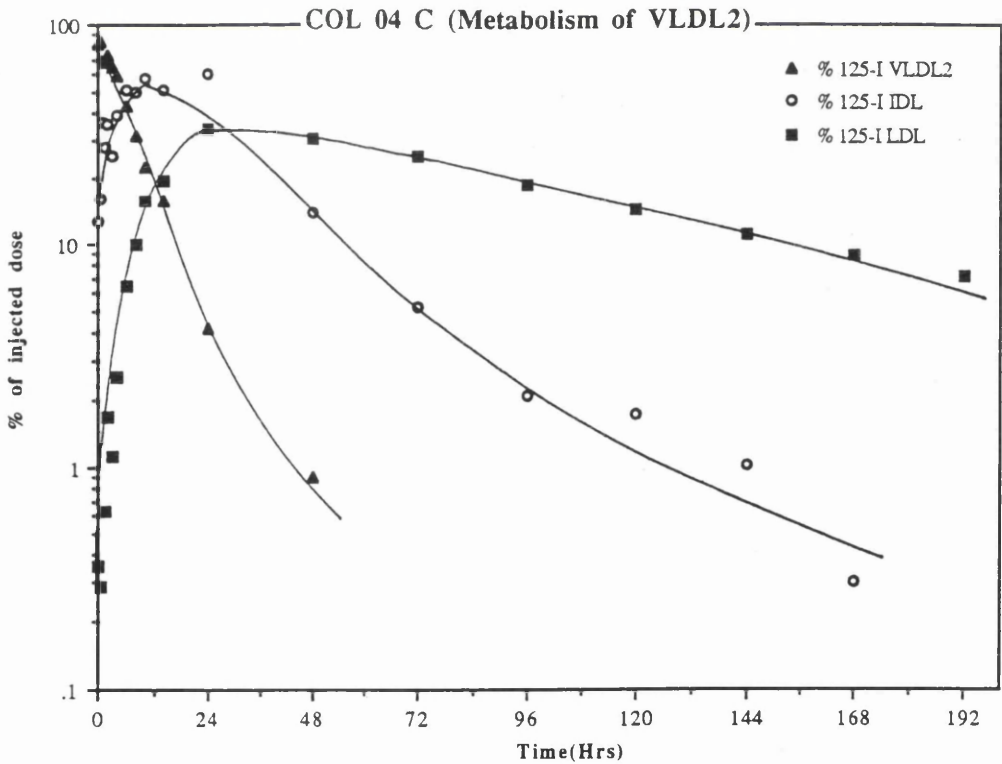
**Figure A-39** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



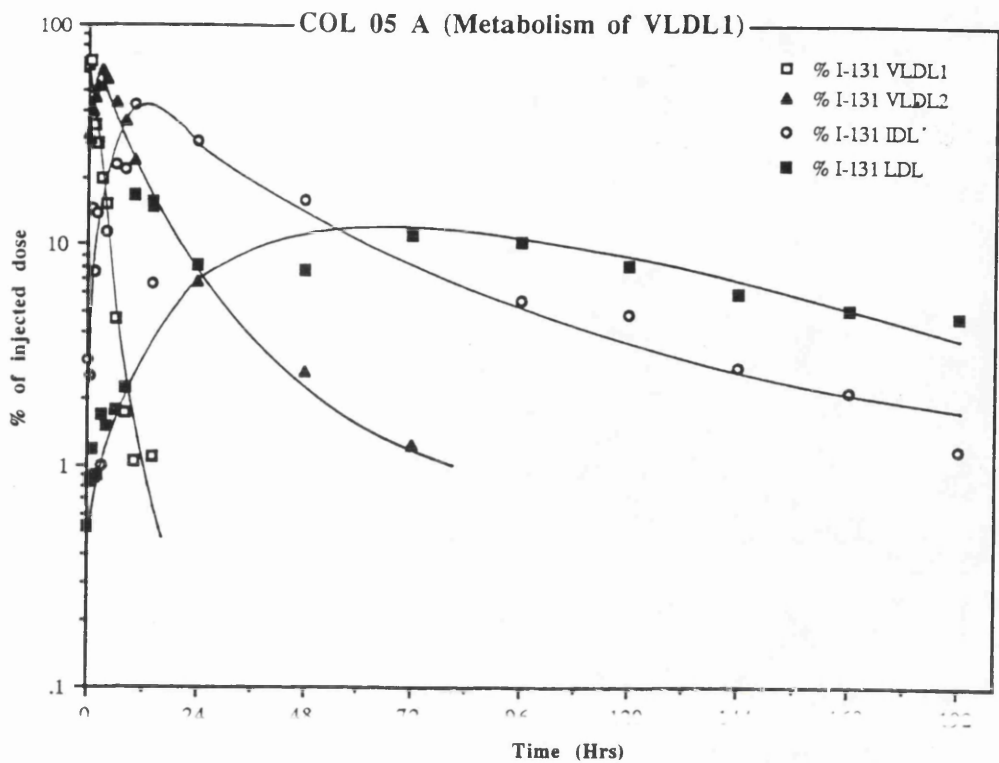
**Figure A-40** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



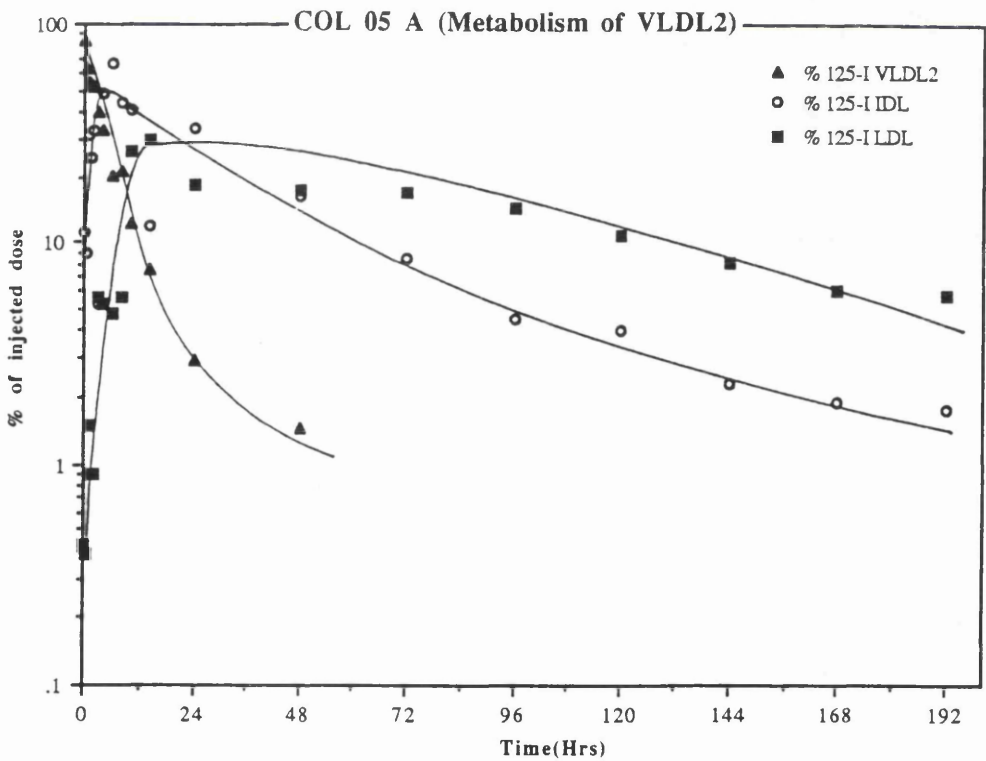
**Figure A-41** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



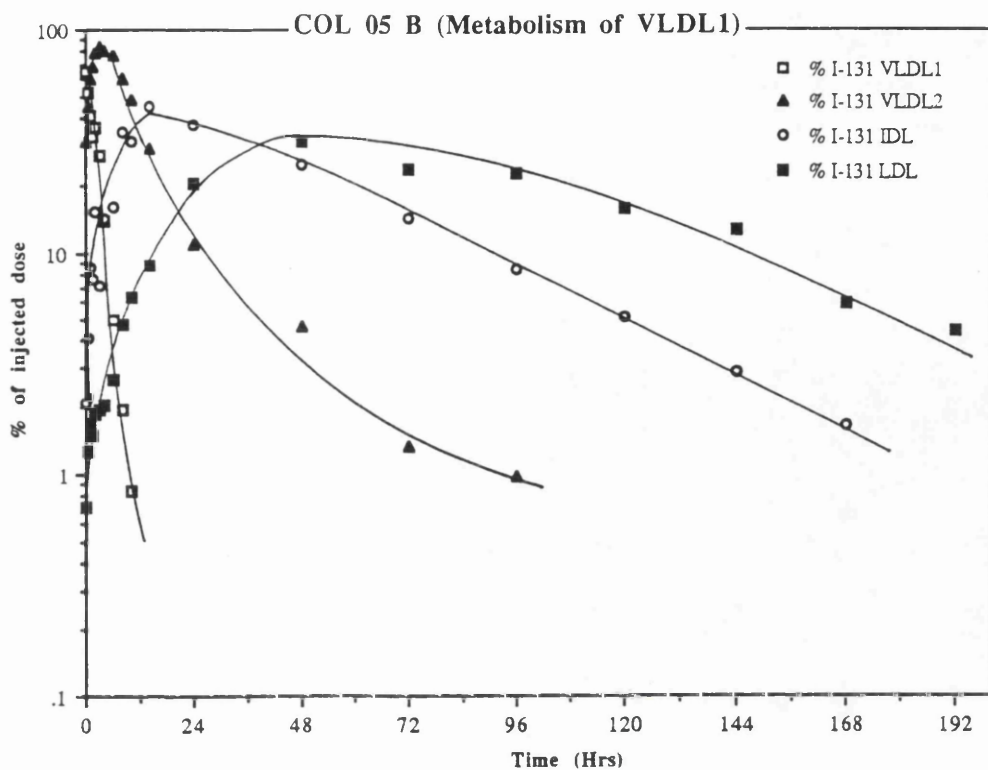
**Figure A-42** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



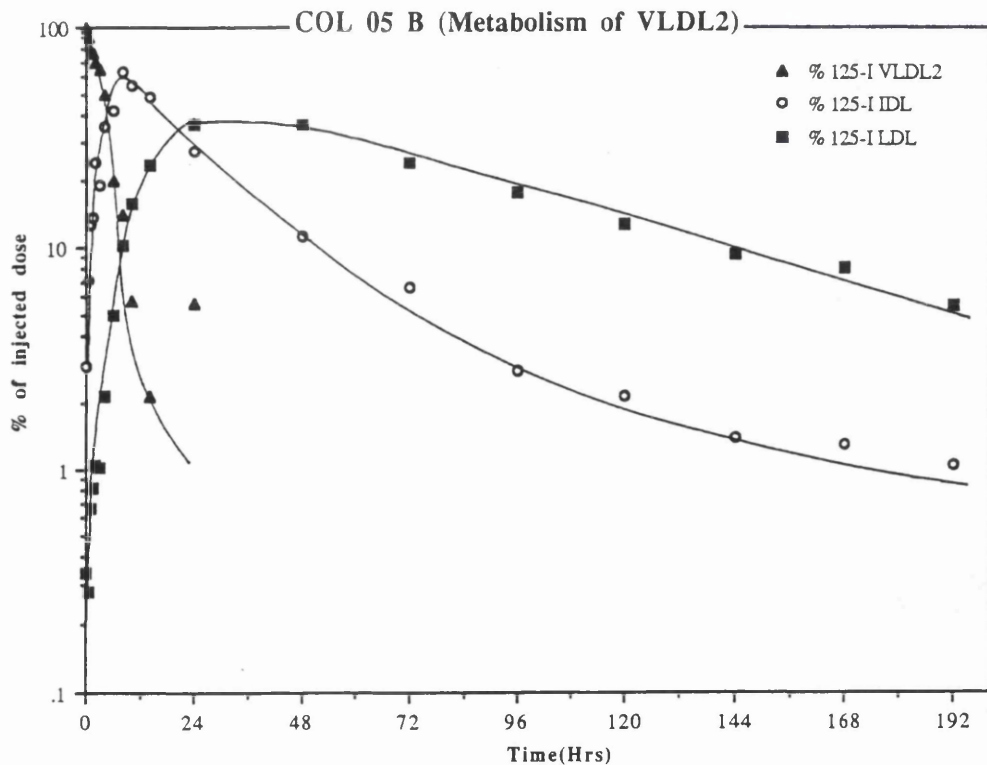
**Figure A-43** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



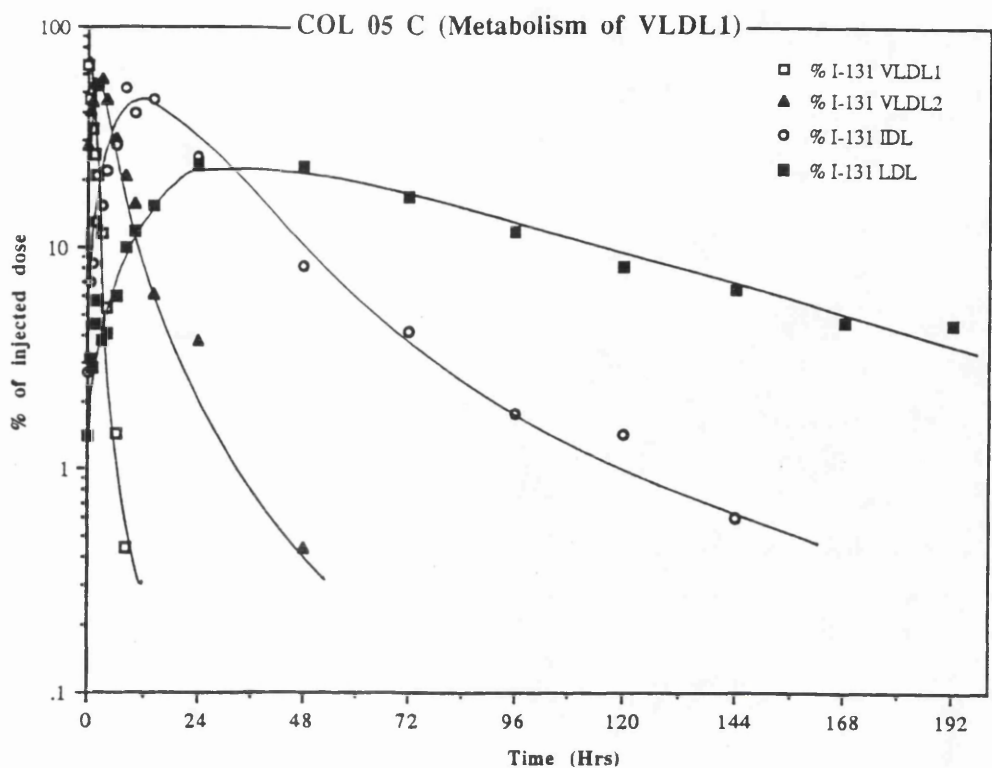
**Figure A-44** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



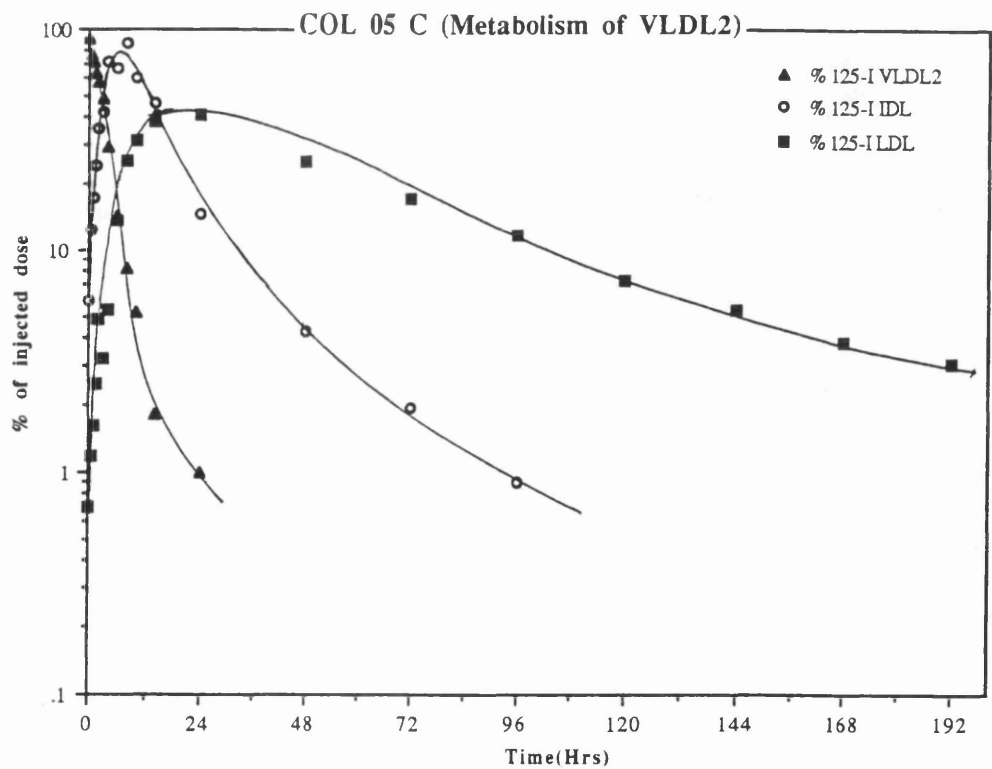
**Figure A-45** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-46** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-47** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-48** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

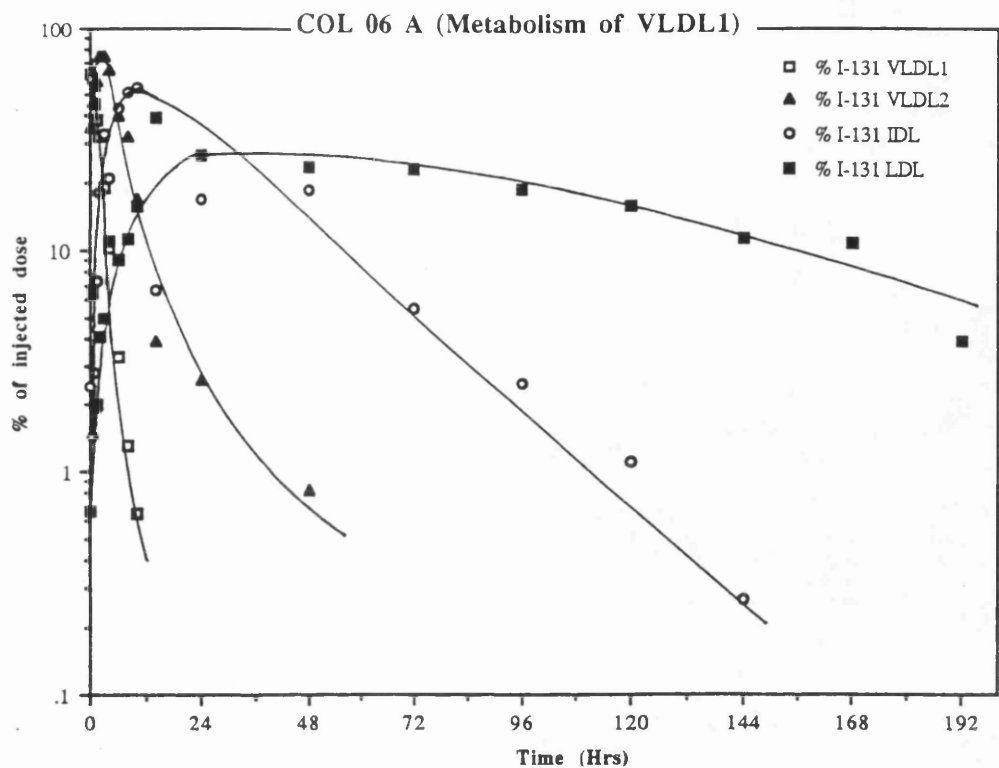


Figure A-49 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

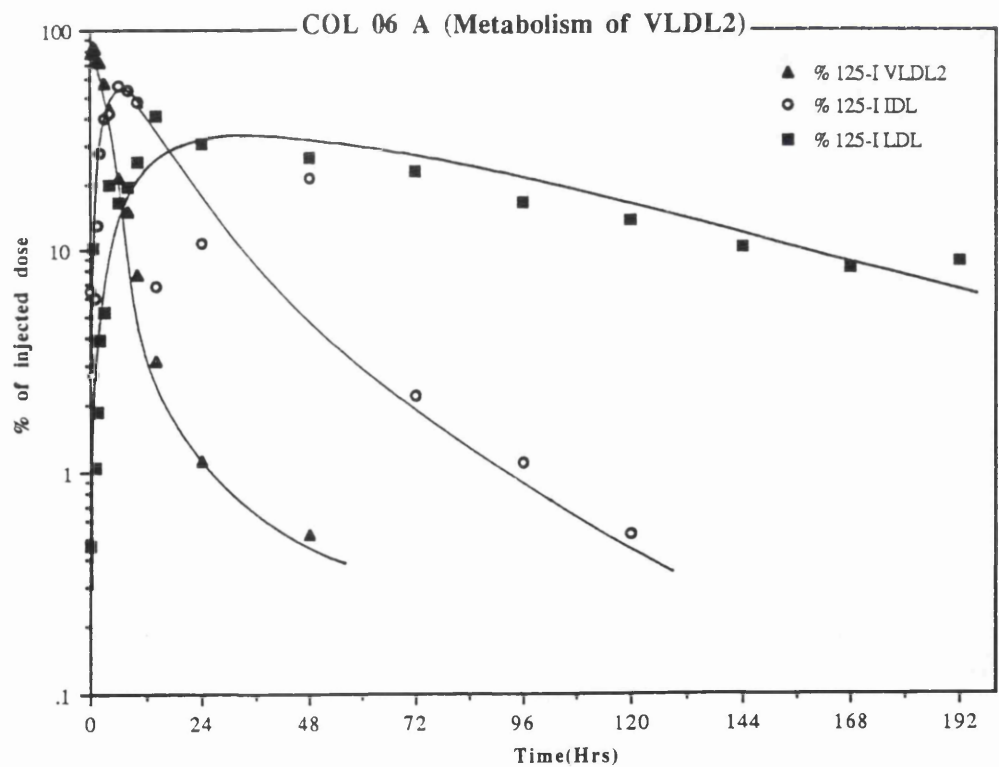
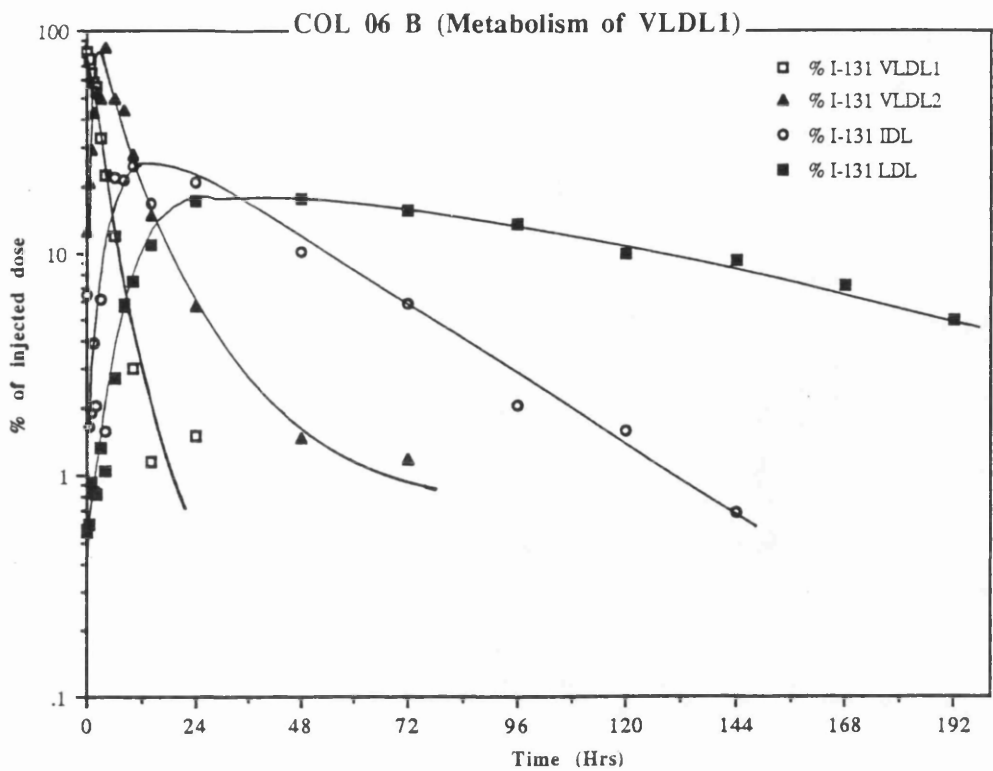
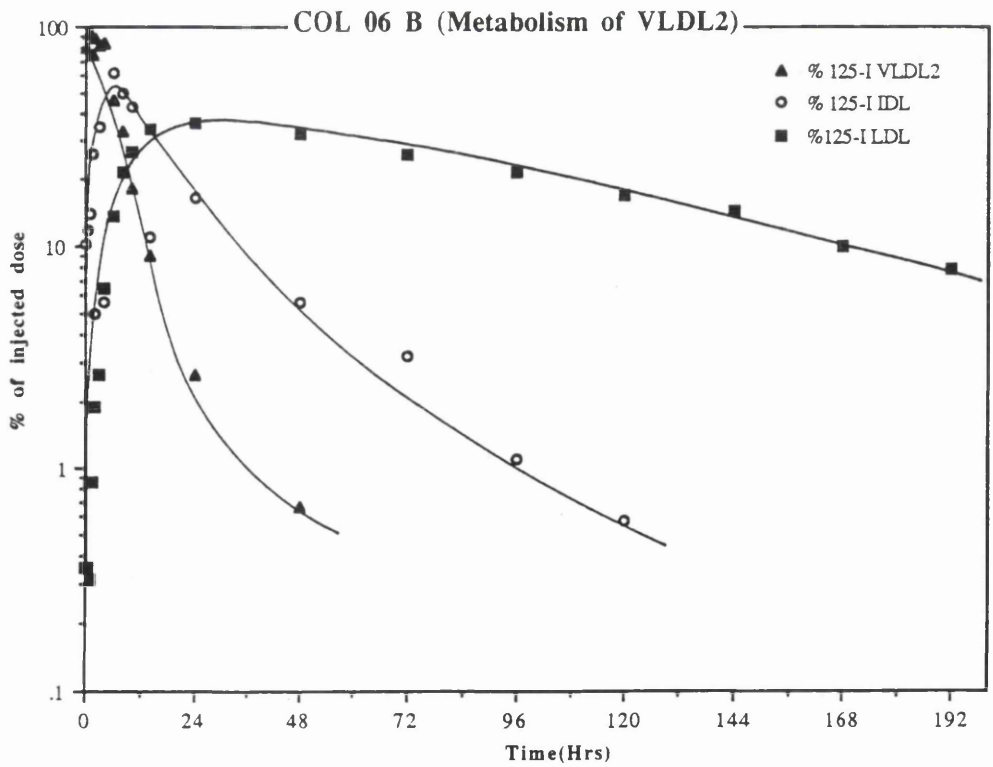


Figure A-50 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

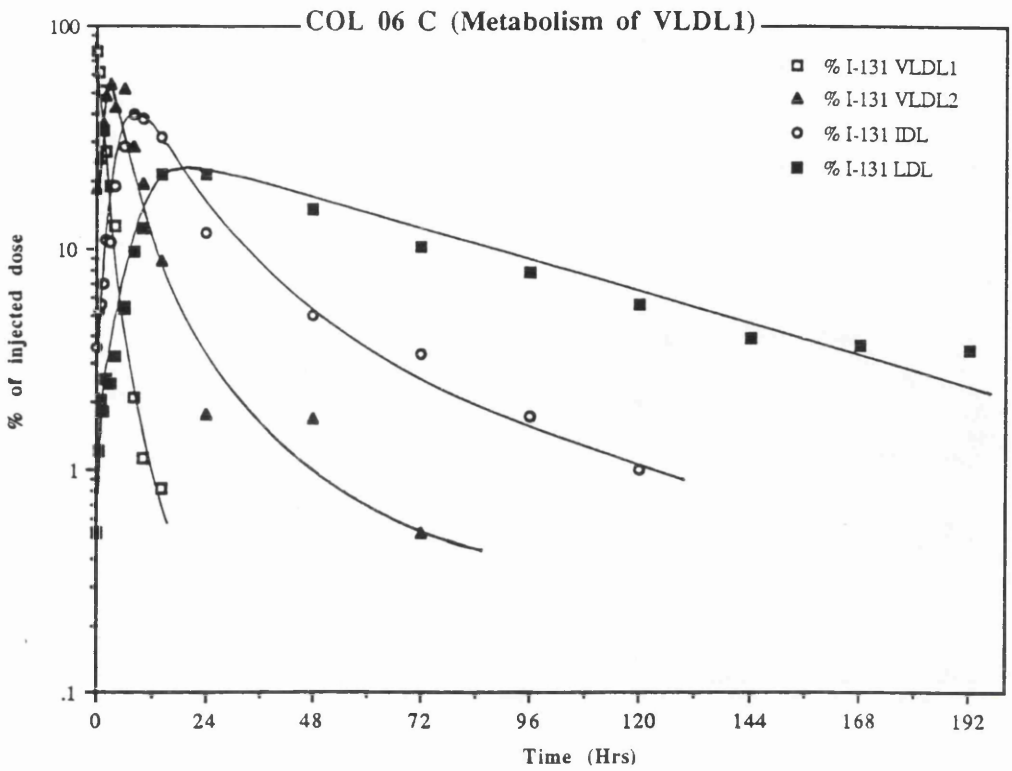


**Figure A-51** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

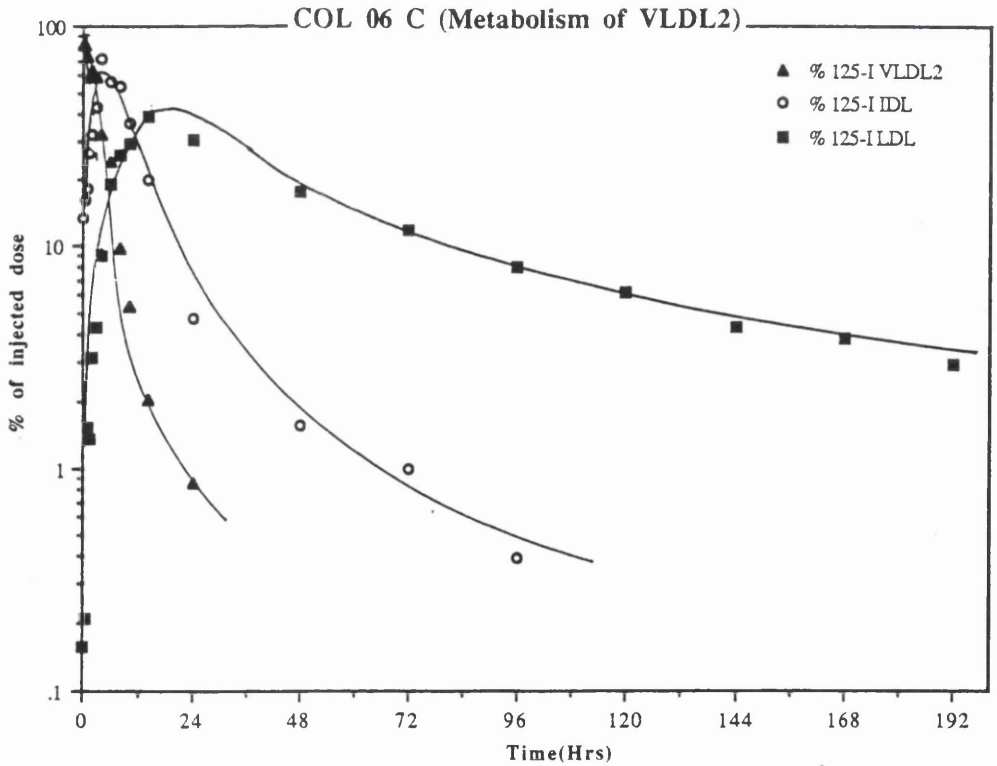


**Figure A-52** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

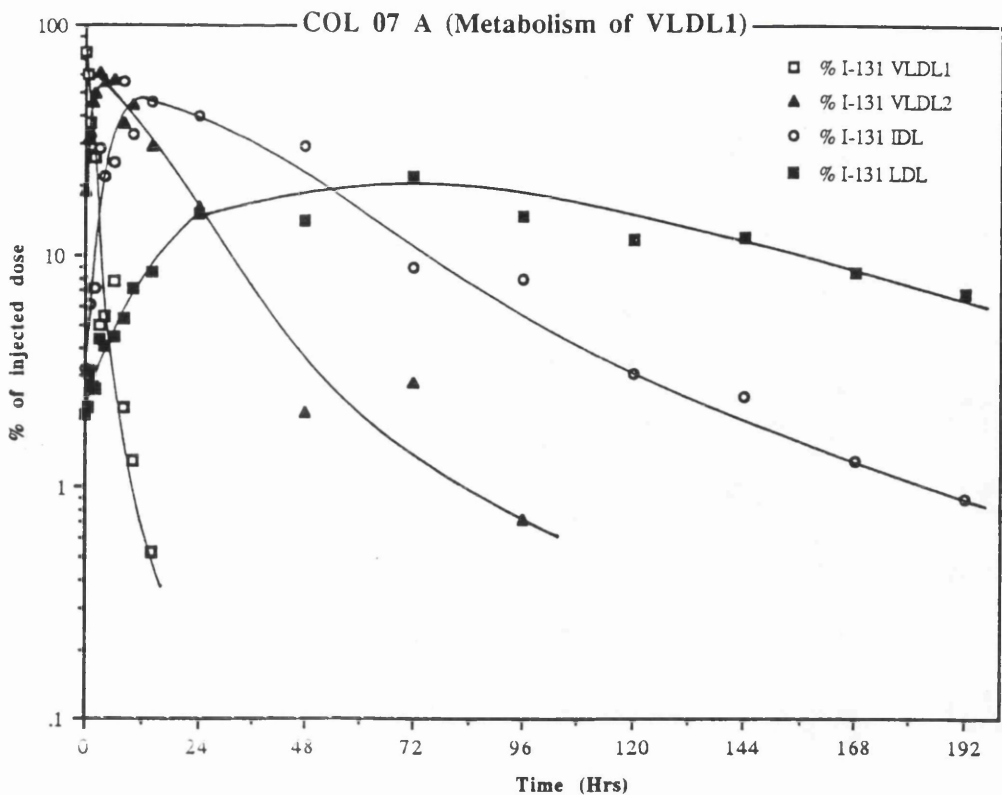




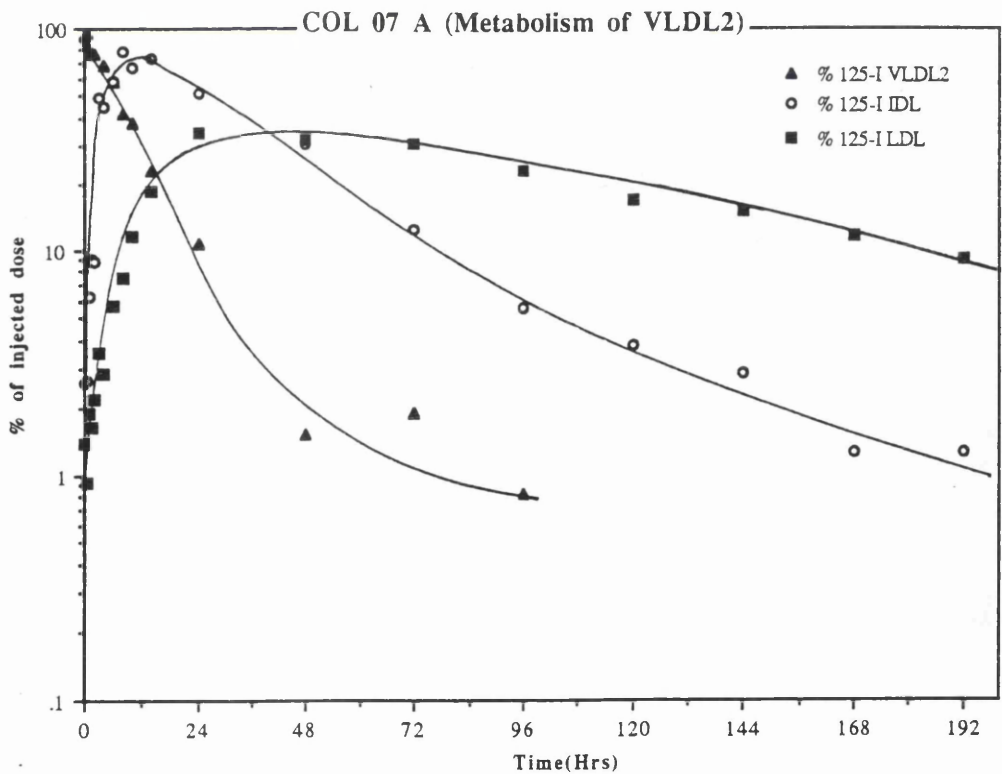
**Figure A-53** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



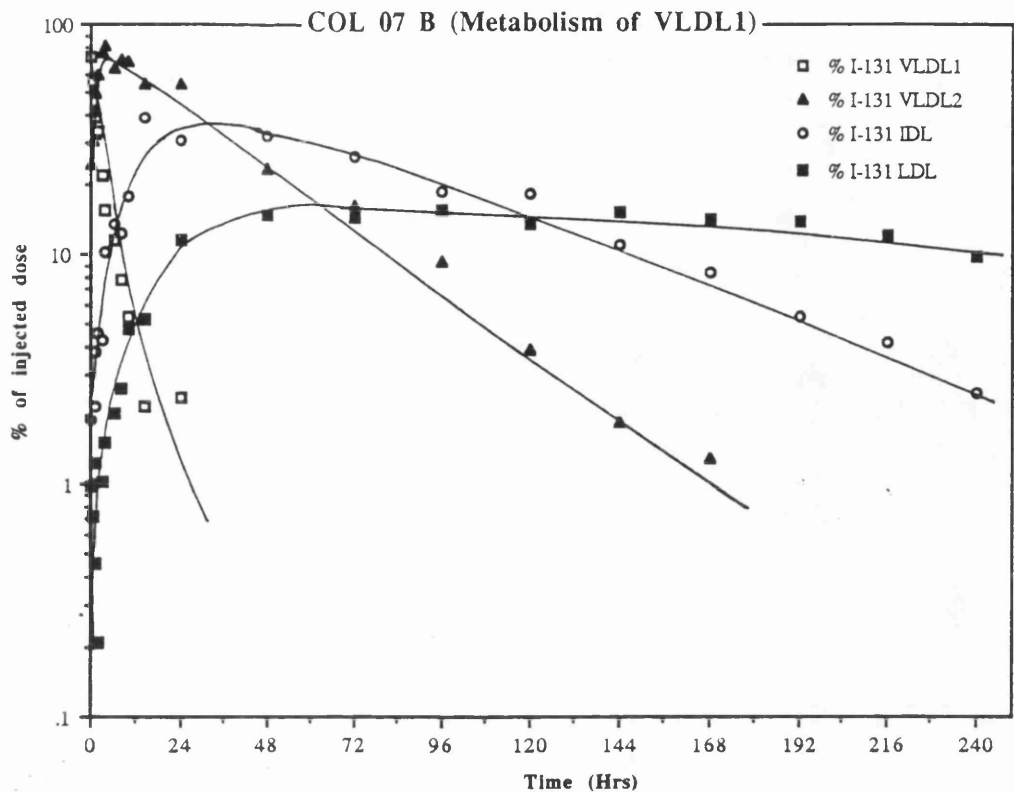
**Figure A-54** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



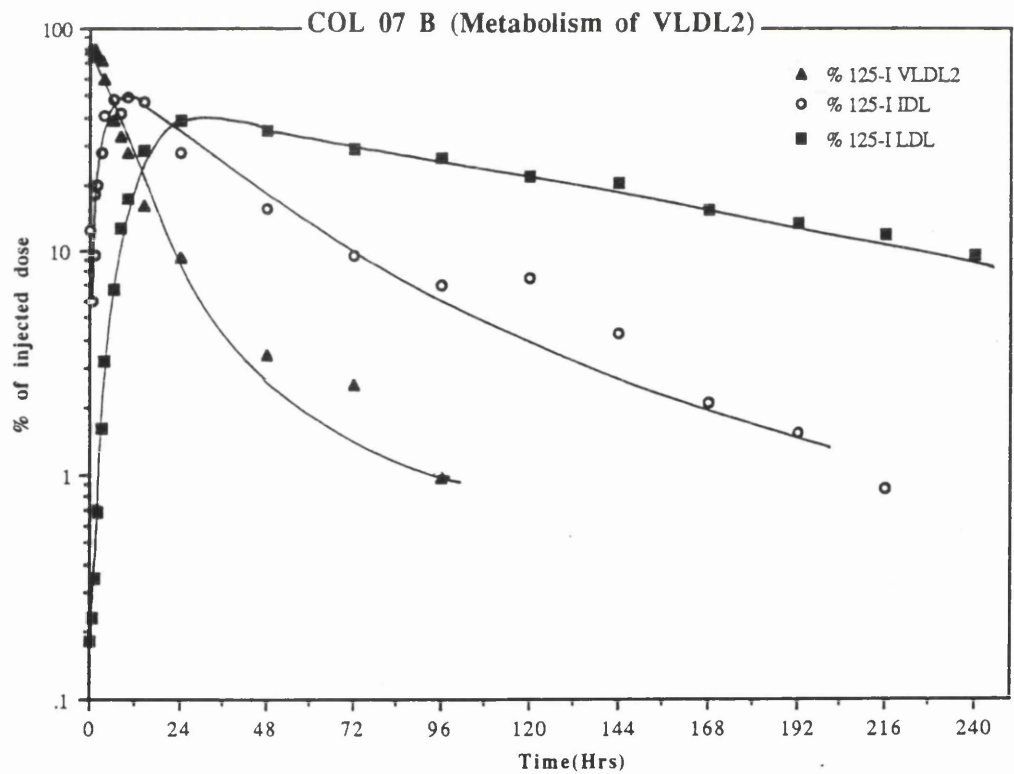
**Figure A-55** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



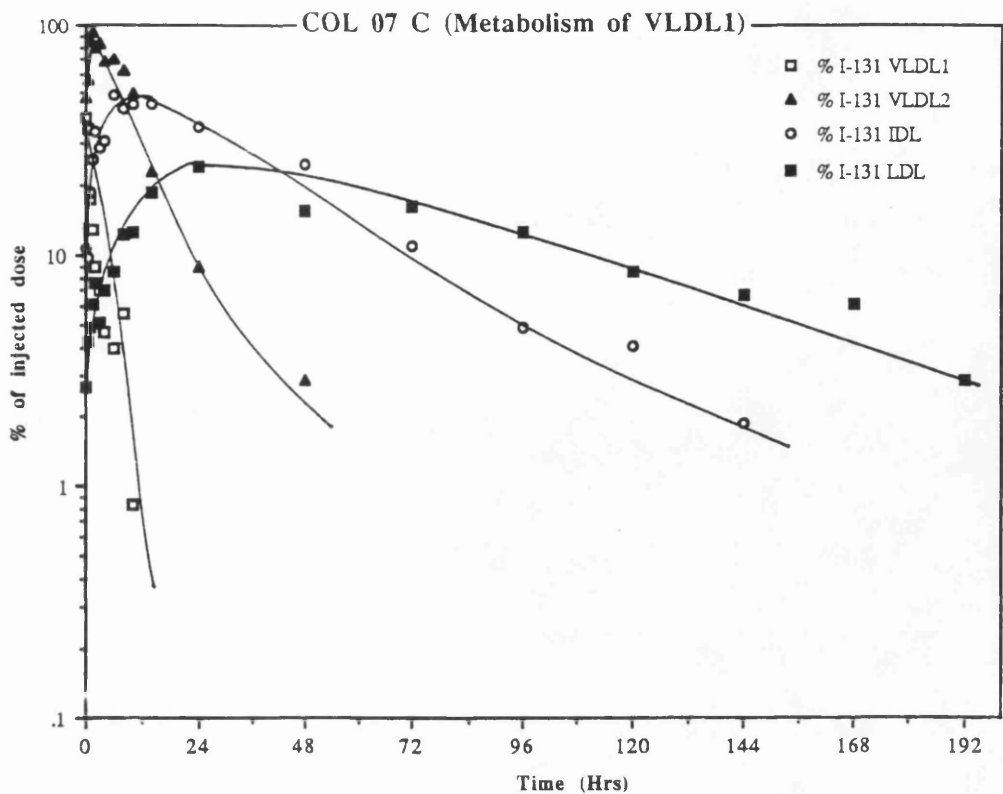
**Figure A-56** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



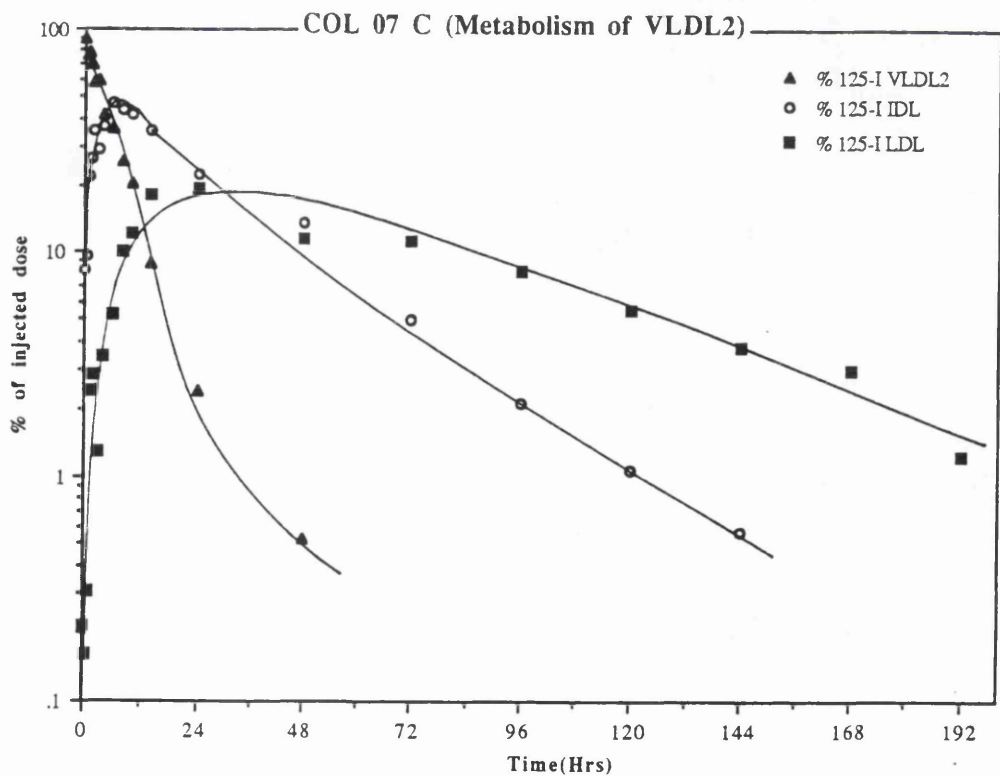
**Figure A-57** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



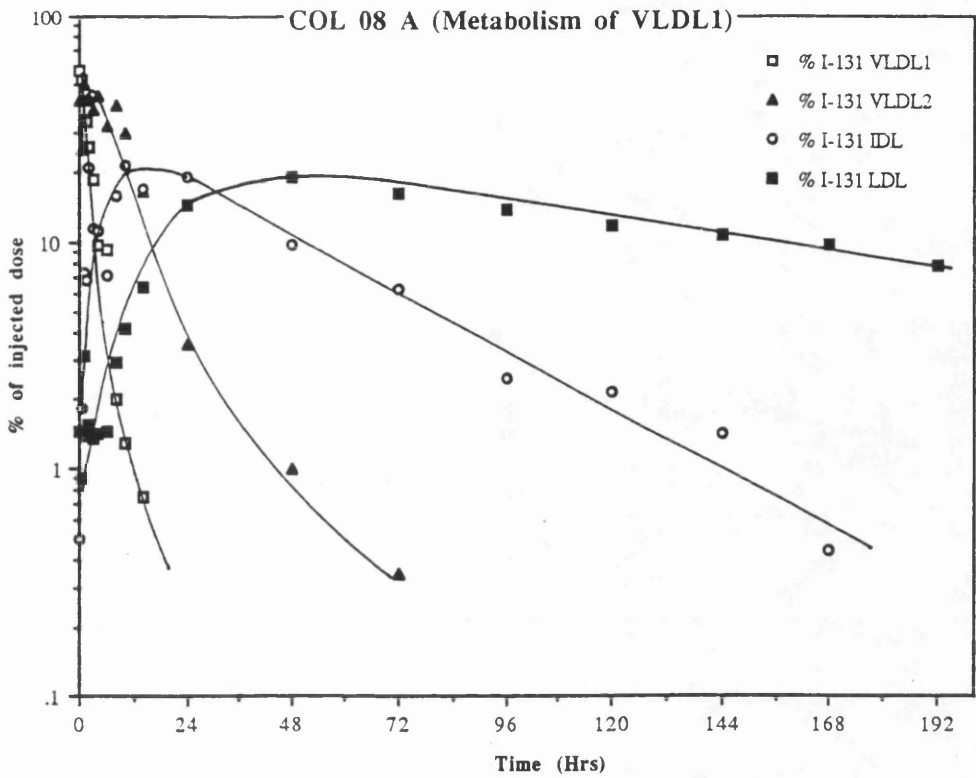
**Figure A-58** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



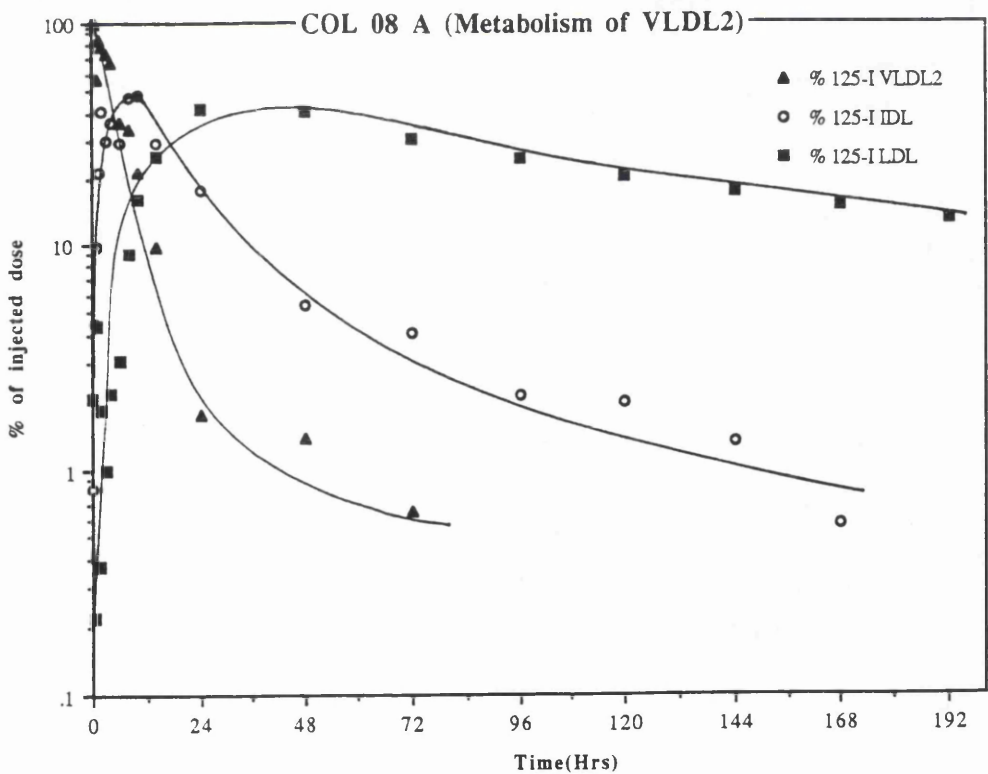
**Figure A-59** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



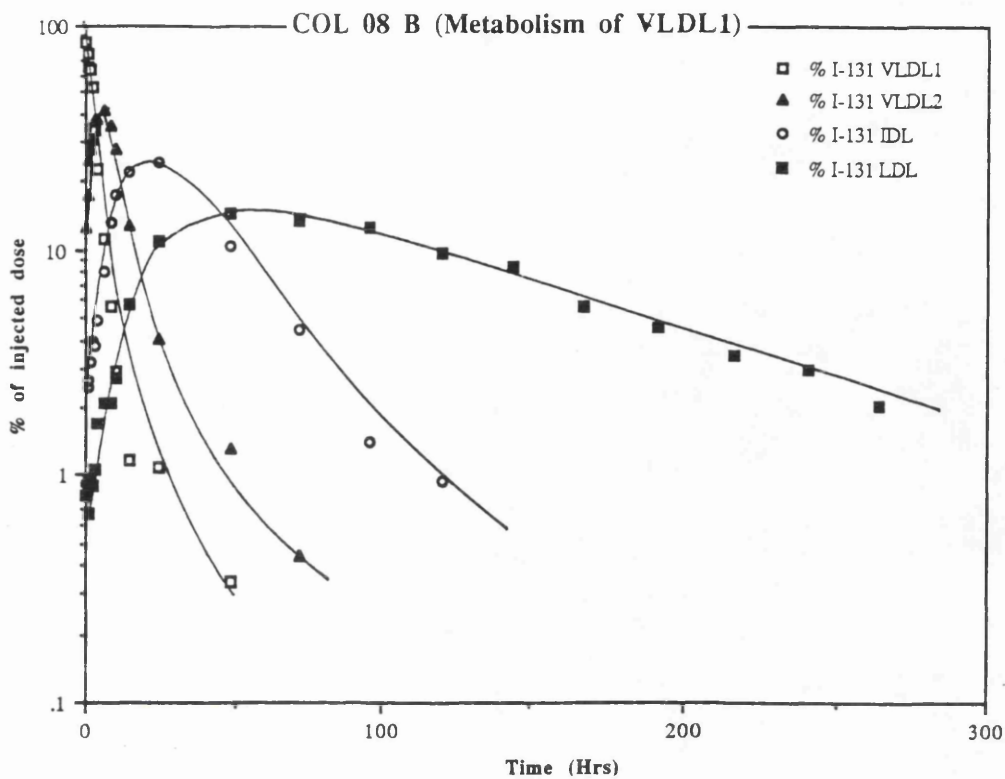
**Figure A-60** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



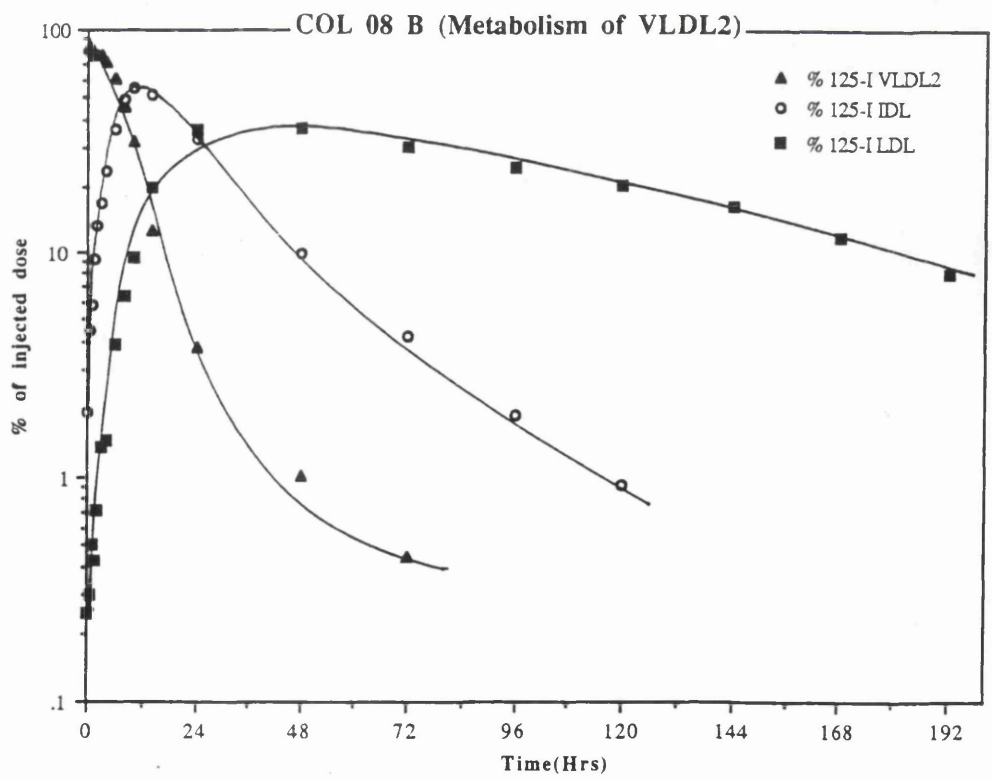
**Figure A-61** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



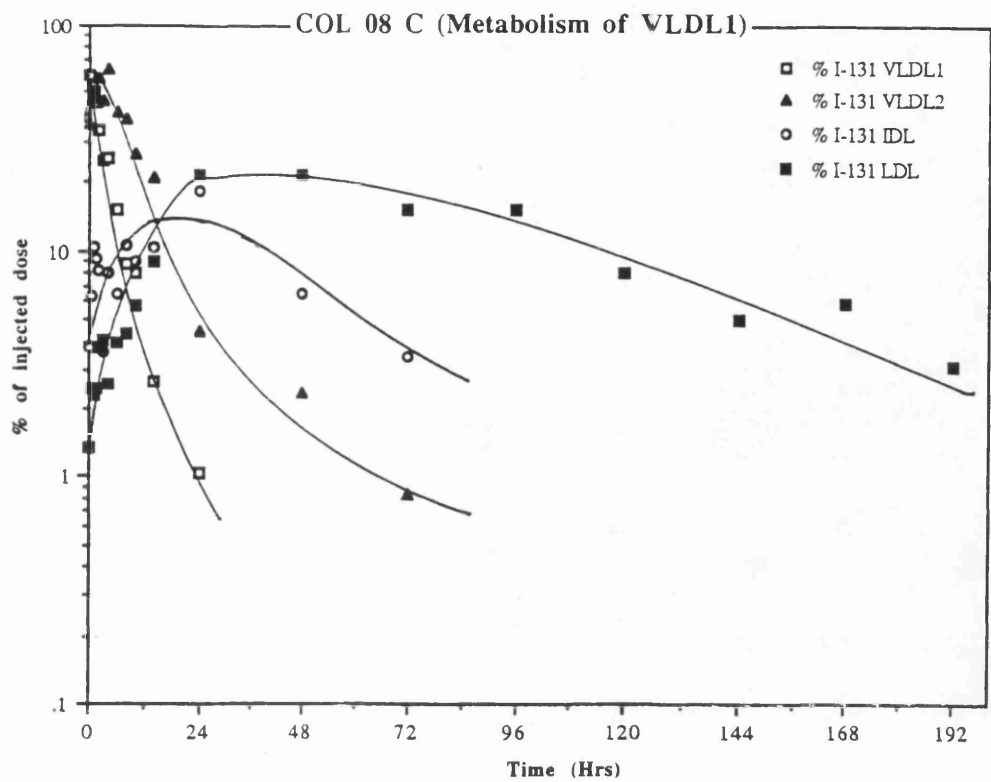
**Figure A-62** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



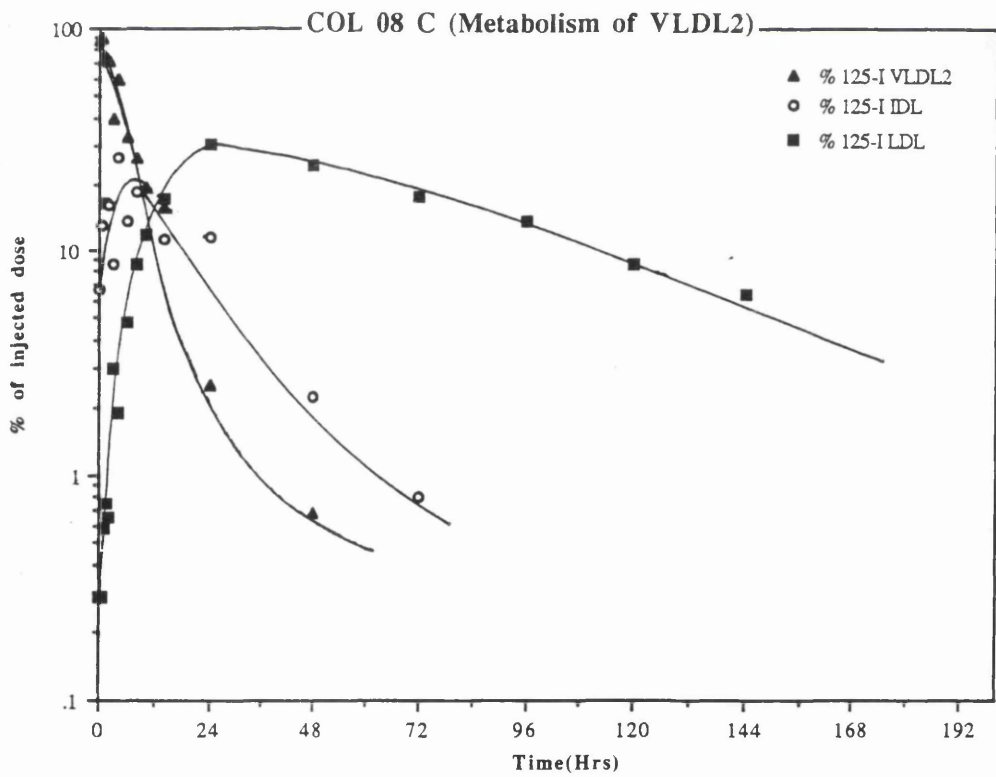
**Figure A-63** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-64** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-65** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous  $[^{131}\text{I}]$ -VLDL<sub>1</sub>.



**Figure A-66** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous  $[^{125}\text{I}]$ -VLDL<sub>2</sub>.

Table A-6. Computed Masses and Rate Constants at Baseline, on Colestipol and on Combined Colestipol and Simvastatin Therapy. VLDL<sub>1</sub>

<i>Before Therapy</i>									
Subject	L(0,1)*	L(2,1)	L(0,12)	L(1,13)	L(12,13)	M(1)†	M(12)	M(13)	U(13)‡
COL 01A	7.17	7.97	0.32	20.39	0.03	50	3	37	758
COL 02A	6.64	9.22	1.30	15.56	0.05	117	5	120	1863
COL 03A	0.00	33.78	0.59	21.45	0.02	16	1	25	541
COL 04A	0.00	14.14	0.28	25.12	0.04	25	2	14	354
COL 05A	7.03	7.92	0.53	14.75	0.02	34	2	35	510
COL 06A	6.99	20.43	1.15	15.43	0.07	58	4	103	1597
COL 07A	3.87	6.72	19.44	33.34	0.45	47	0	15	505
COL 08A	9.02	4.92	1.67	14.75	0.24	87	12	82	1187
Mean	5.09	13.14	3.16	20.10	0.12	54	4	54	914
SEM	1.22	3.43	2.33	2.32	0.05	12	1	15	200
<i>On Colestipol</i>									
COL 01B	7.47	11.09	0.79	16.47	0.03	57	3	64	1061
COL 02B	0.00	10.96	1.99	12.52	0.25	111	12	97	1240
COL 03B	0.00	9.07	0.42	9.46	0.02	44	2	42	400
COL 04B	0.97	7.60	0.68	11.90	0.02	54	1	39	464
COL 05B	0.00	11.42	2.22	13.58	0.02	37	0	31	423
COL 06B	0.76	10.91	0.86	10.54	0.02	112	4	124	1310
COL 07B	3.98	5.37	0.35	9.69	0.02	27	2	26	253
COL 08B	6.38	4.93	0.59	15.82	0.02	186	5	133	2109
Mean	2.45	8.92	0.99	12.50	0.05	79	4	70	908
SEM	1.09	0.94	0.25	0.94	0.03	19	1	15	226
<i>On Combination Therapy</i>									
COL 01C	14.67	2.20	1.43	19.11	0.06	68	3	60	1150
COL 02C	2.96	12.98	0.41	17.27	0.02	34	2	31	543
COL 03C	2.09	10.33	0.90	22.63	0.06	56	2	31	697
COL 04C	2.62	8.09	2.94	32.98	0.02	55	0	18	589
COL 05C	1.45	13.61	11.64	40.08	6.77	29	6	11	506
COL 06C	2.00	11.07	0.54	14.37	0.02	55	2	50	720
COL 07C	2.46	25.43	0.15	24.71	0.02	15	3	16	419
COL 08C	0.00	5.99	0.17	43.08	0.02	185	4	26	1132
Mean	3.53	11.21	2.27	26.78	0.87	63	3	30	720
SEM	1.62	2.43	1.38	3.79	0.84	19	1	6	98

\*Rate constants, L(destination, source) are in units, d<sup>-1</sup> † Masses M( ) are in mg. ‡ U( ) represents *de novo* synthesis of apo B into a compartment



Table A-7. Computed Masses and Rate Constants at Baseline, on Colestipol and on Combined Colestipol and Simvastatin Therapy. VLDL<sub>2</sub>

Before Therapy										
Subject	L(4,2)	L(6,2)	L(0,6)	L(0,4)	L(8,4)	L(11,4)	L(9,4)	L(0,5)	L(7,5)	L(10,5)
COL 01A	7.31	0.04	0.47	0.98	3.28	0.30	0.00	2.35	1.06	0.00
COL 02A	24.00	0.12	0.62	2.78	3.39	0.12	0.88	0.00	5.65	0.00
COL 03A	24.00	0.10	0.67	0.00	4.25	0.59	0.72	0.98	3.59	0.00
COL 04A	24.00	0.18	0.41	0.00	1.76	0.24	1.42	0.00	5.08	0.00
COL 05A	24.00	0.40	0.60	1.94	1.02	0.15	0.14	1.72	5.14	0.00
COL 06A	8.16	0.05	0.75	1.65	6.16	0.24	0.17	1.50	3.12	0.74
COL 07A	9.74	0.45	0.71	0.00	1.47	0.04	0.26	0.00	2.81	0.00
COL 08A	5.37	0.17	0.96	0.70	2.16	0.02	0.22	0.00	3.54	0.00
Mean	15.82	0.19	0.65	1.01	2.94	0.21	0.48	0.82	3.75	0.09
SEM	3.12	0.06	0.06	0.37	0.60	0.06	0.17	0.34	0.53	0.09
On Colestipol										
COL 01B	18.15	0.08	0.57	2.80	2.59	0.02	0.46	0.52	4.92	0.00
COL 02B	9.84	0.02	0.43	4.29	2.91	0.13	0.50	1.59	1.70	0.00
COL 03B	4.84	0.05	0.45	0.00	3.16	0.11	0.49	0.62	1.77	0.30
COL 04B	17.55	13.90	2.67	0.00	1.23	0.06	0.03	2.46	1.72	0.00
COL 05B	10.45	0.00	0.35	0.69	2.22	0.09	0.57	3.90	3.37	0.00
COL 06B	19.49	0.06	0.55	2.95	1.86	0.00	0.59	0.00	3.04	0.00
COL 07B	6.19	0.29	0.31	0.05	0.59	0.07	0.62	0.00	3.04	0.00
COL 08B	4.70	0.02	0.36	1.84	3.58	0.00	1.50	1.53	1.32	0.00
Mean	11.40	1.80	0.71	1.58	2.27	0.06	0.60	1.33	2.61	0.04
SEM	2.19	1.73	0.28	0.58	0.36	0.02	0.15	0.48	0.43	0.04
On Combination Therapy										
COL 01C	12.94	0.36	1.32	0.00	24.00	0.00	1.53	0.00	7.82	0.00
COL 02C	10.32	0.00	1.51	0.00	24.00	0.72	2.22	5.72	3.48	0.00
COL 03C	24.00	0.00	0.51	0.00	3.26	0.02	0.31	0.15	3.38	0.00
COL 04C	24.00	0.26	0.56	1.17	1.30	0.00	0.45	0.00	3.45	0.00
COL 05C	9.37	0.01	0.20	0.33	5.98	0.00	3.08	0.62	5.46	0.00
COL 06C	7.42	0.07	0.79	1.29	6.62	0.00	2.12	0.00	6.82	0.00
COL 07C	24.00	0.00	0.10	0.11	0.15	0.76	2.08	1.67	4.42	0.00
COL 08C	3.09	6.17	9.15	0.00	2.13	0.00	0.28	0.00	4.96	0.00
Mean	14.39	0.86	1.77	0.36	8.43	0.19	1.51	1.02	4.97	0.00
SEM	2.98	0.76	1.07	0.19	3.49	0.12	0.37	0.70	0.59	0.00

**Table A-8.**Computed Masses and Rate Constants at Baseline, on Colestipol and on Combined Colestipol and Simvastatin Therapy. VLDL<sub>2</sub> (contd)

<i>Before Therapy</i>					
Subject	M(2)	M(4)	M(6)	M(5)	U(5)
COL 01A	54	87	4	142	484
COL 02A	45	151	8	55	311
COL 03A	22	96	3	113	517
COL 04A	15	103	6	130	661
COL 05A	11	82	7	162	1111
COL 06A	145	144	10	54	289
COL 07A	31	173	20	95	267
COL 08A	77	134	14	272	964
Mean	50	121	9	128	576
SEM	16	12	2	25	112
<i>On Colestipol</i>					
COL 01B	34	107	5	116	631
COL 02B	123	155	5	103	339
COL 03B	82	106	10	211	568
COL 04B	13	174	68	367	1534
COL 05B	41	119	0	97	705
COL 06B	63	226	7	236	716
COL 07B	22	103	21	242	736
COL 08B	194	132	9	168	479
Mean	72	140	16	193	714
SEM	22	15	8	32	127
<i>On Combination Therapy</i>					
COL 01C	11	6	3	61	477
COL 02C	42	16	0	48	442
COL 03C	24	161	0	207	731
COL 04C	19	152	8	164	565
COL 05C	42	42	2	82	499
COL 06C	82	61	7	177	1207
COL 07C	15	119	0	185	1128
COL 08C	122	157	82	264	1309
Mean	45	89	13	149	795
SEM	14	23	10	27	128

Table A-9. Computed Masses and Rate Constants at Baseline, on Colestipol and on Combined Colestipol and Simvastatin Therapy. IDL

<i>Before Therapy</i>									
Subject	L(0,8)	L(11,8)	L(0,9)	L(0,7)	L(10,7)	M(8)	M(9)	M(7)	
COL 01A	0.13	0.43	0.07	0.00	2.24	501	5	67	
COL 02A	0.02	1.61	0.49	2.22	2.28	313	268	70	
COL 03A	0.26	1.15	0.47	0.53	1.30	289	146	221	
COL 04A	0.24	0.34	0.57	0.29	1.07	313	257	487	
COL 05A	0.00	0.49	0.16	0.52	0.80	171	72	634	
COL 06A	0.24	0.89	0.36	1.54	2.16	786	69	45	
COL 07A	0.30	0.50	0.31	0.00	1.12	316	147	238	
COL 08A	0.09	0.62	0.31	1.25	1.68	408	96	329	
Mean	0.16	0.75	0.34	0.79	1.58	387	133	261	
SEM	0.04	0.15	0.06	0.28	0.21	66	33	75	
<i>On Colestipol</i>									
COL 01B	1.19	0.48	0.42	1.40	0.76	166	116	263	
COL 02B	0.23	1.36	0.47	0.33	2.79	284	165	56	
COL 03B	0.79	0.67	0.48	0.75	1.23	229	107	190	
COL 04B	0.54	0.25	0.23	0.11	0.88	269	20	633	
COL 05B	0.33	0.82	0.35	0.37	1.53	230	196	173	
COL 06B	0.00	1.36	0.58	1.97	1.29	309	232	220	
COL 07B	0.23	0.57	0.33	0.73	1.09	76	195	404	
COL 08B	0.00	1.01	0.78	0.00	1.35	471	254	164	
Mean	0.41	0.82	0.46	0.71	1.37	254	161	263	
SEM	0.15	0.14	0.06	0.24	0.22	41	27	63	
<i>On Combination Therapy</i>									
COL 01C	0.28	0.75	0.42	1.52	0.93	133	21	195	
COL 02C	0.71	1.87	0.32	0.00	9.57	150	113	18	
COL 03C	1.64	0.79	0.30	1.23	1.72	216	164	237	
COL 04C	0.12	0.74	0.44	0.89	0.83	231	156	330	
COL 05C	1.24	1.51	0.72	0.74	1.38	92	181	211	
COL 06C	0.78	2.12	0.80	3.21	1.75	138	159	243	
COL 07C	0.00	0.34	1.08	0.95	0.98	52	230	422	
COL 08C	0.00	2.22	0.52	11.83	0.54	151	86	106	
Mean	0.60	1.29	0.58	2.55	2.21	145	139	220	
SEM	0.22	0.26	0.10	1.37	1.06	21	23	44	

Table A-10. Computed Masses and Rate Constants at Baseline, on Colestipol and on Combined Colestipol and Simvastatin Therapy. LDL

<i>Before Therapy</i>			
Subject	L(0,11)	L(0,10)	M(11) M(10)
COL 01A	0.39	0.23	874 658
COL 02A	0.19	0.35	2736 456
COL 03A	0.27	0.31	1421 911
COL 04A	0.19	0.30	694 1737
COL 05A	0.29	0.32	330 1591
COL 06A	0.25	0.42	2942 329
COL 07A	0.26	0.39	627 679
COL 08A	0.22	0.26	1188 2096
Mean	0.26	0.32	1352 1057
SEM	0.02	0.02	346 233
<i>On Colestipol</i>			
COL 01B	0.19	0.34	422 581
COL 02B	0.25	0.49	1629 321
COL 03B	0.25	0.30	657 980
COL 04B	0.20	0.22	397 2596
COL 05B	0.32	0.16	632 1659
COL 06B	0.28	0.18	1501 1540
COL 07B	0.12	0.23	405 1903
COL 08B	0.28	0.20	1696 1097
Mean	0.24	0.27	917 1335
SEM	0.02	0.04	206 262
<i>On Combination Therapy</i>			
COL 01C	0.36	0.32	282 572
COL 02C	0.42	0.20	696 851
COL 03C	0.28	0.57	719 629
COL 04C	0.21	0.32	803 854
COL 05C	0.27	0.42	522 694
COL 06C	0.36	0.37	815 1142
COL 07C	0.30	0.50	363 831
COL 08C	0.32	0.04	1037 1413
Mean	0.32	0.34	655 873
SEM	0.02	0.06	89 99

*Appendix 4*

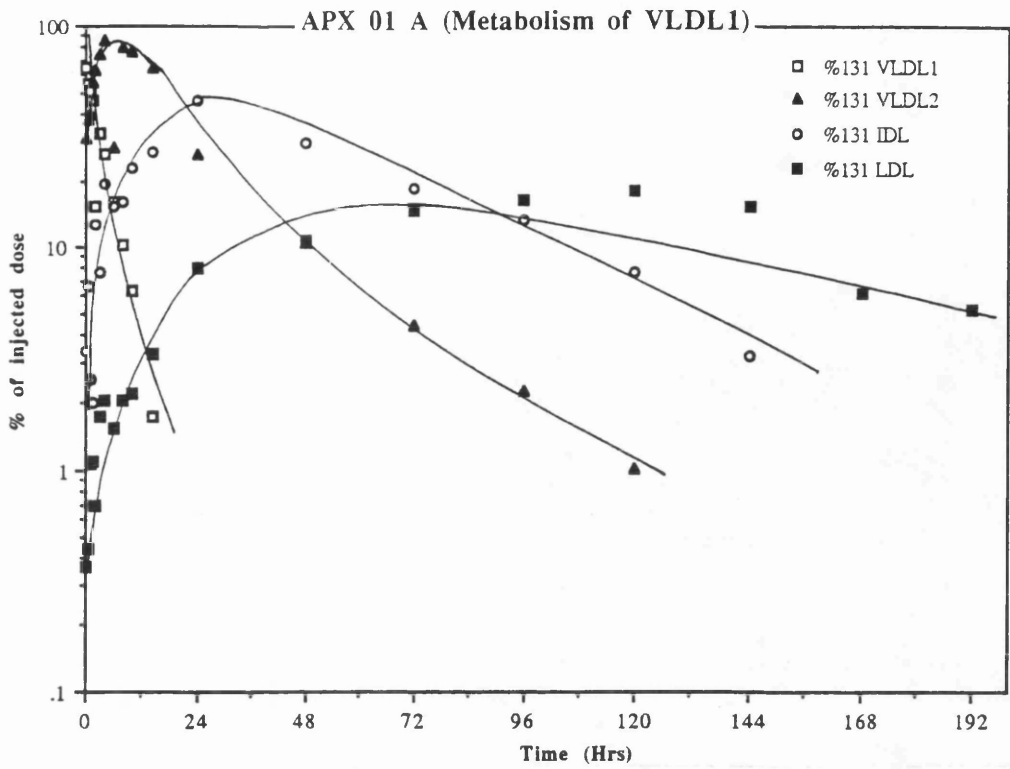


Figure A-67 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

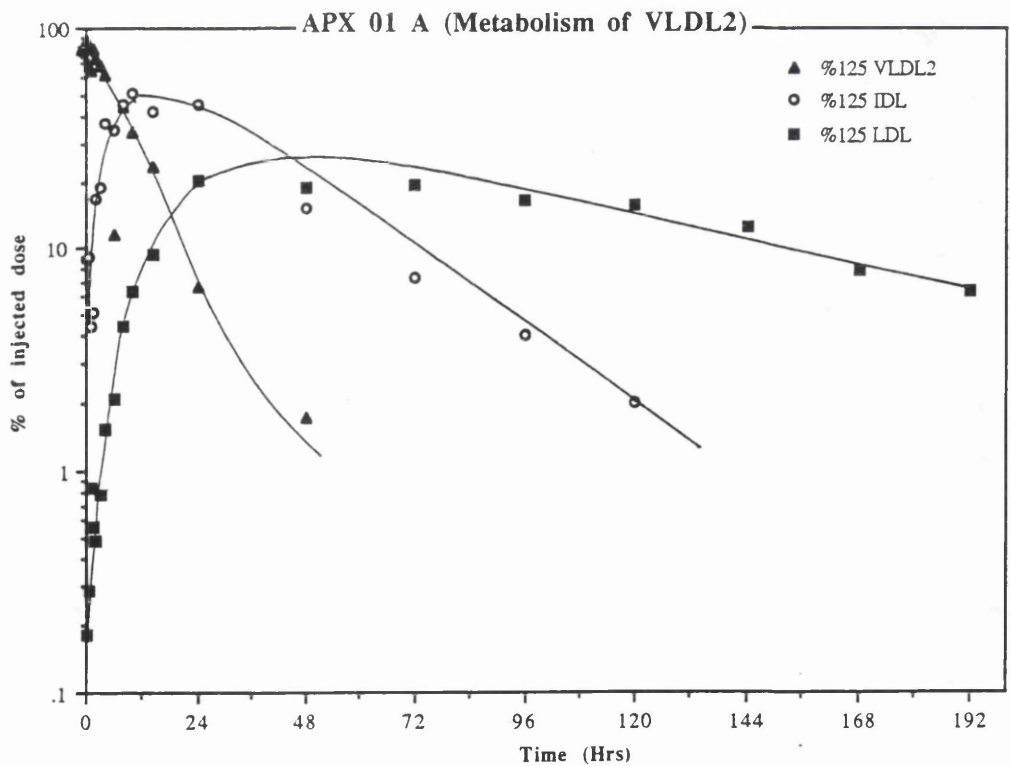


Figure A-68 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

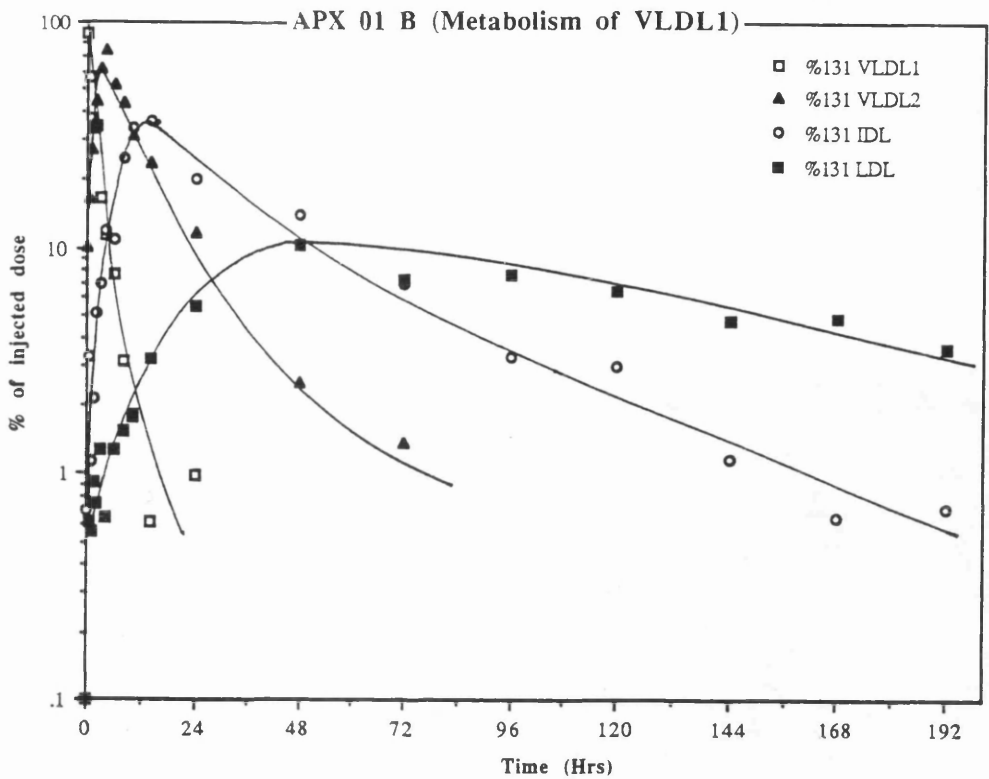


Figure A-69 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{131}$ I]-VLDL<sub>1</sub>.

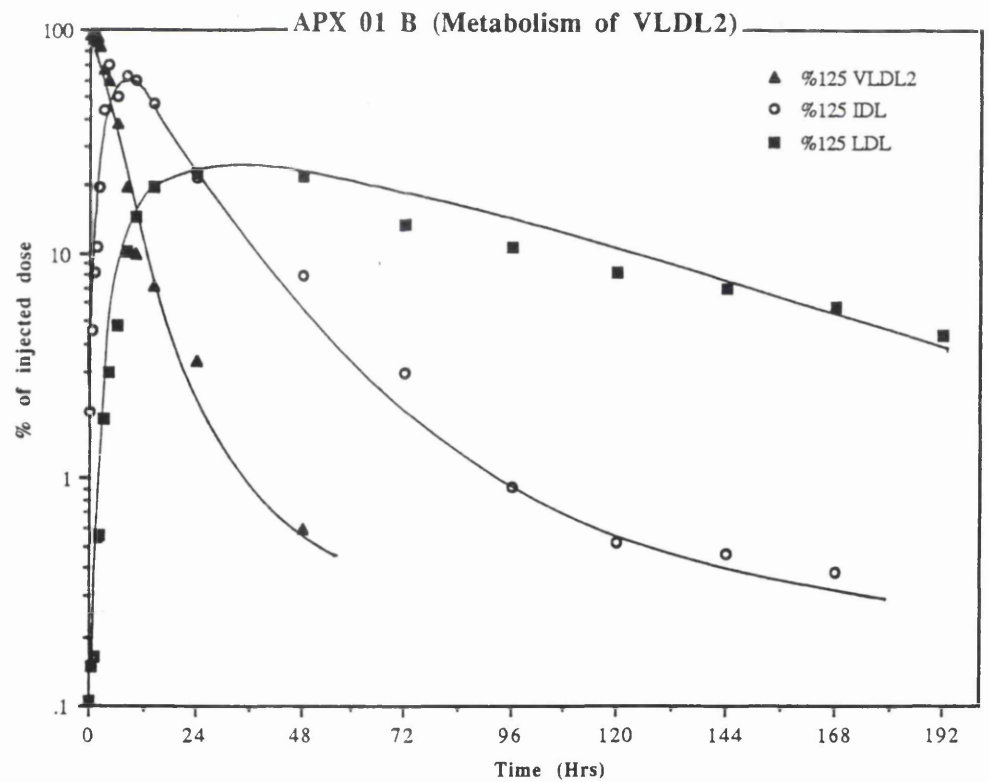


Figure A-70 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{125}$ I]-VLDL<sub>2</sub>.

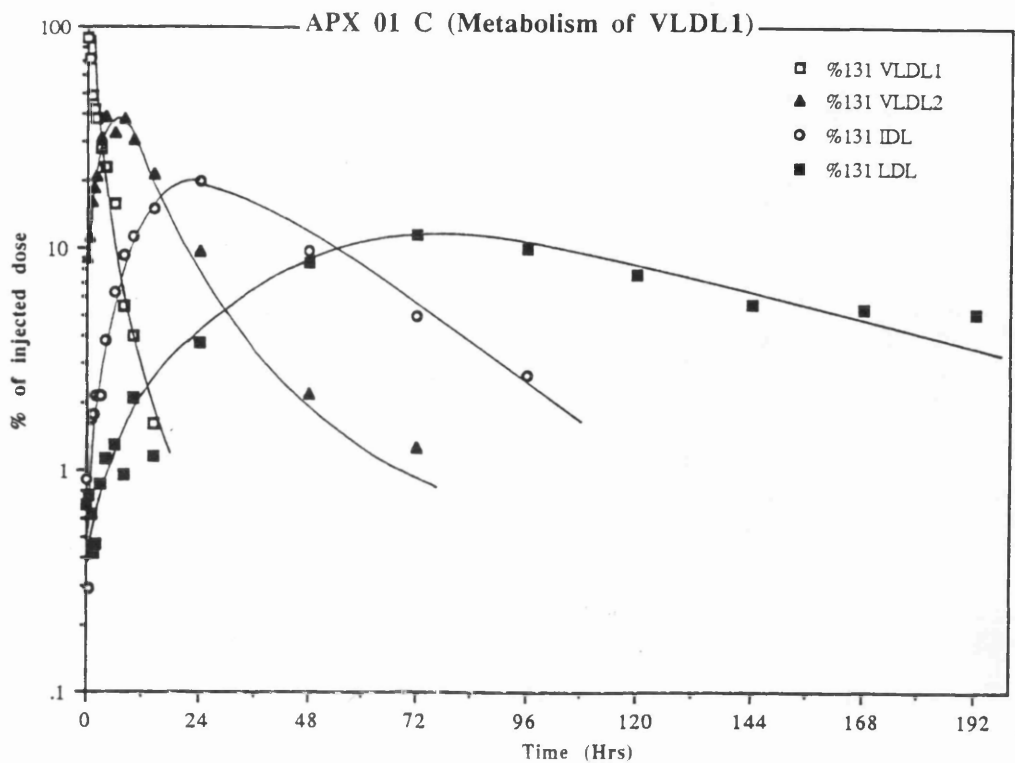


Figure A-71 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

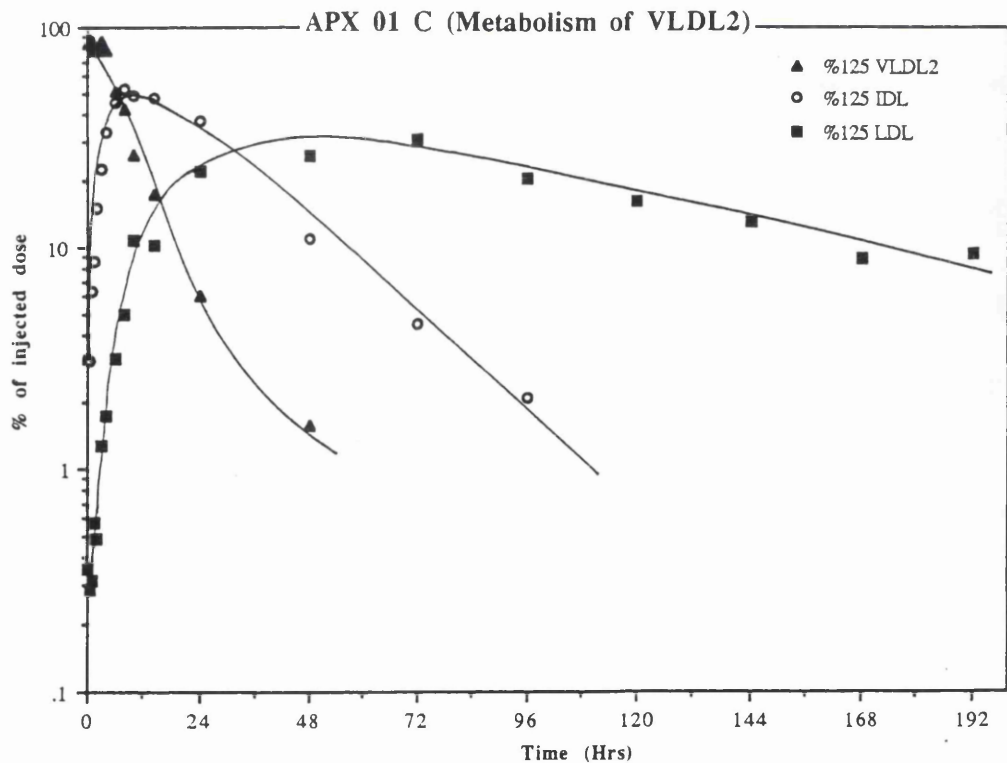


Figure A-72 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



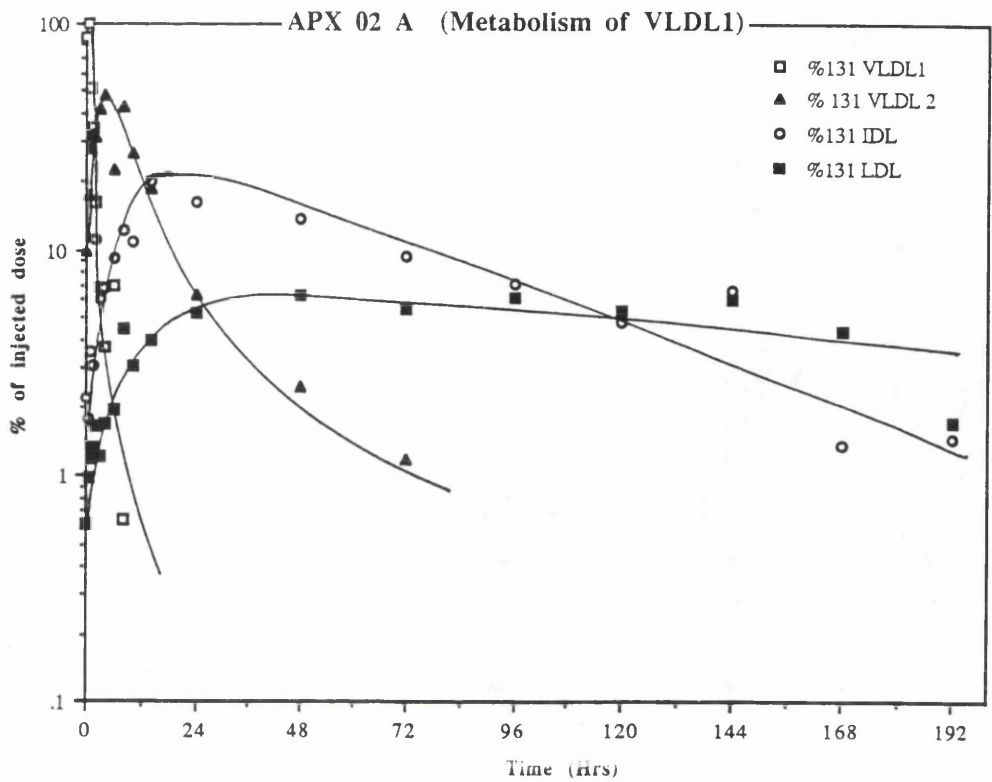


Figure A-73 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

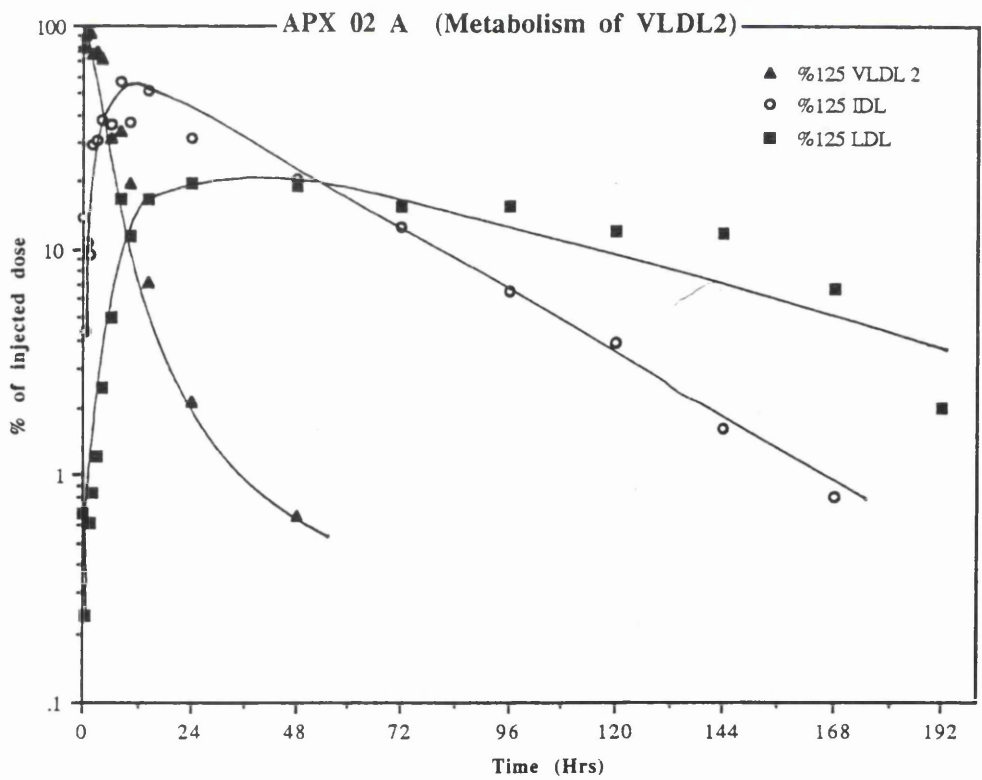
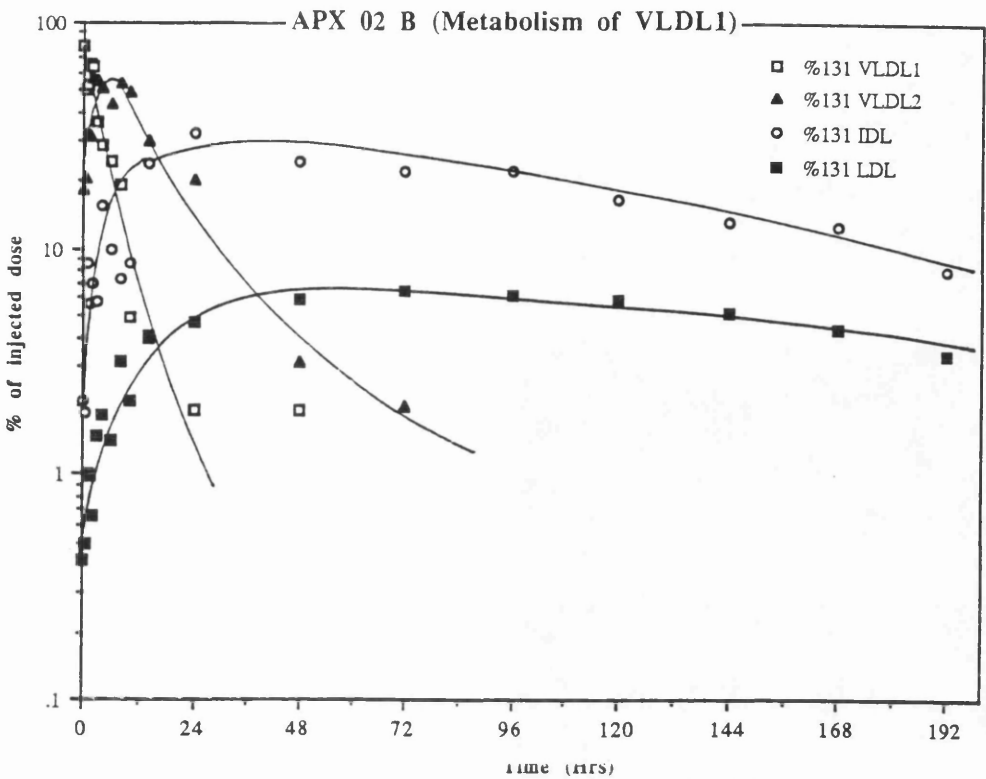
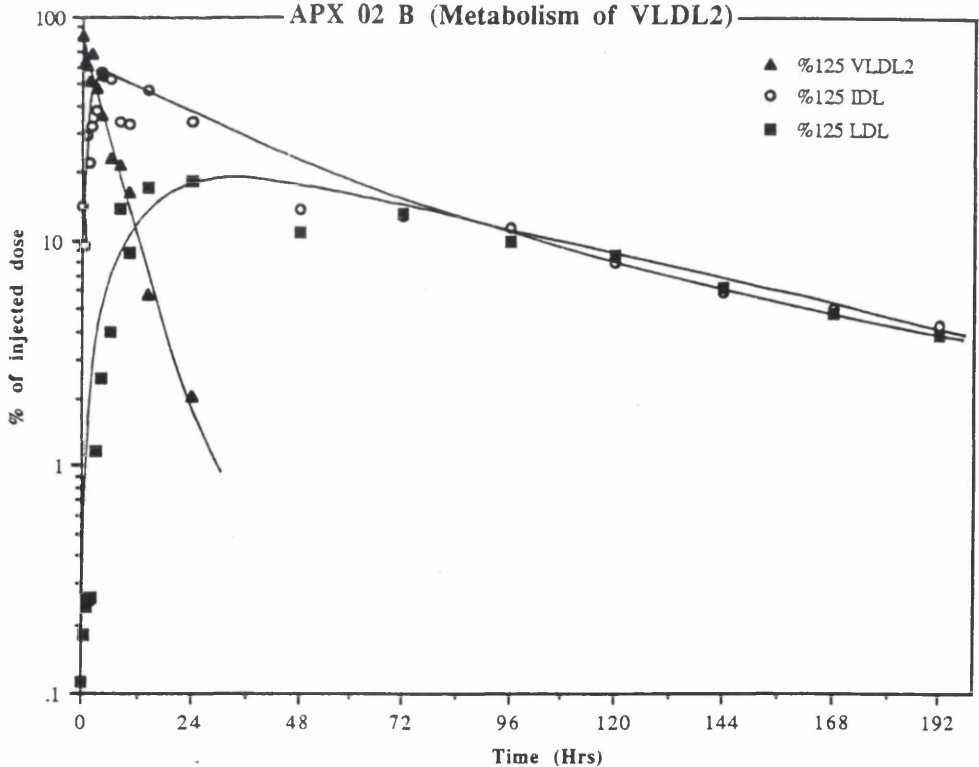


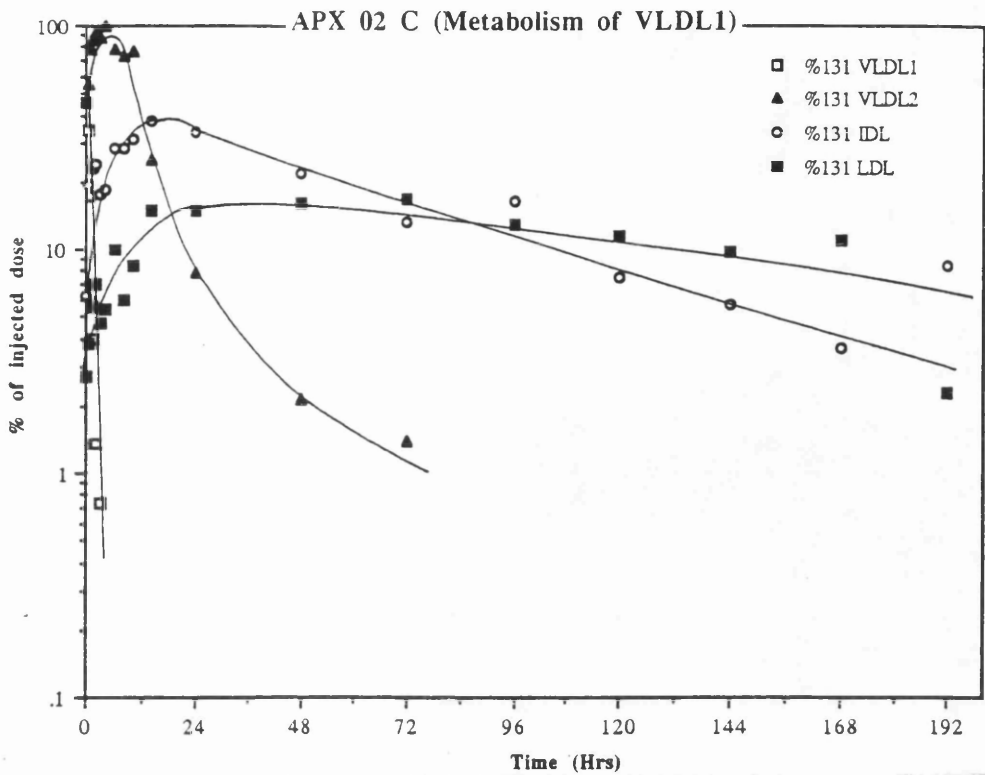
Figure A-74 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



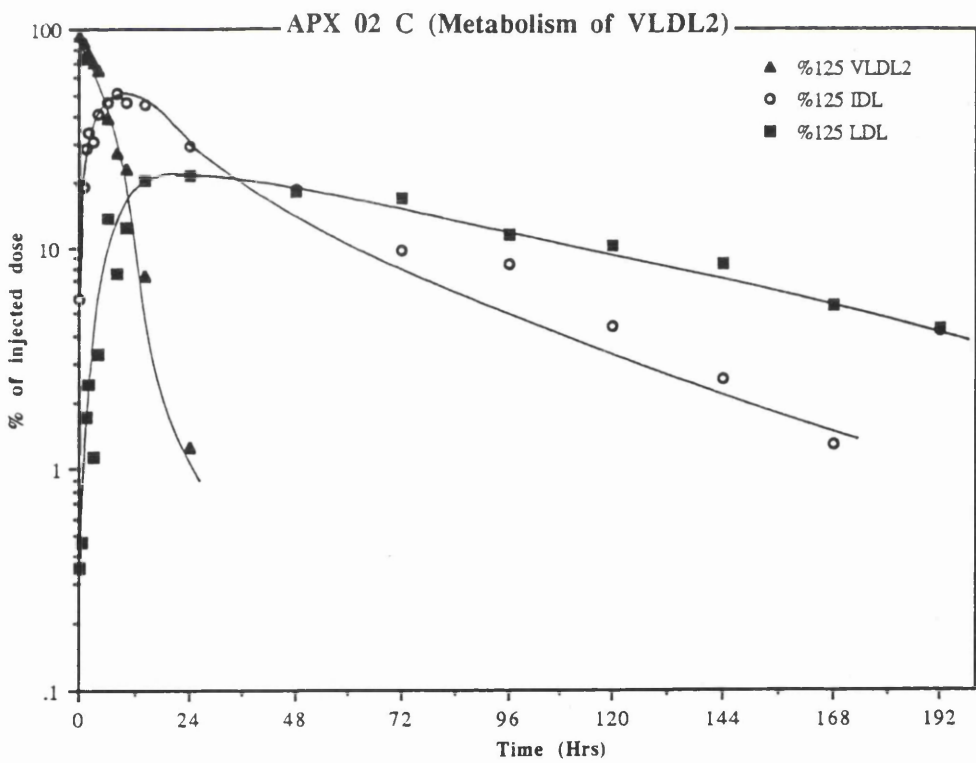
**Figure A-75** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{131}\text{I}$ ]-VLDL<sub>1</sub>.



**Figure A-76** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{125}\text{I}$ ]-VLDL<sub>2</sub>.



**Figure A-77** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-78** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

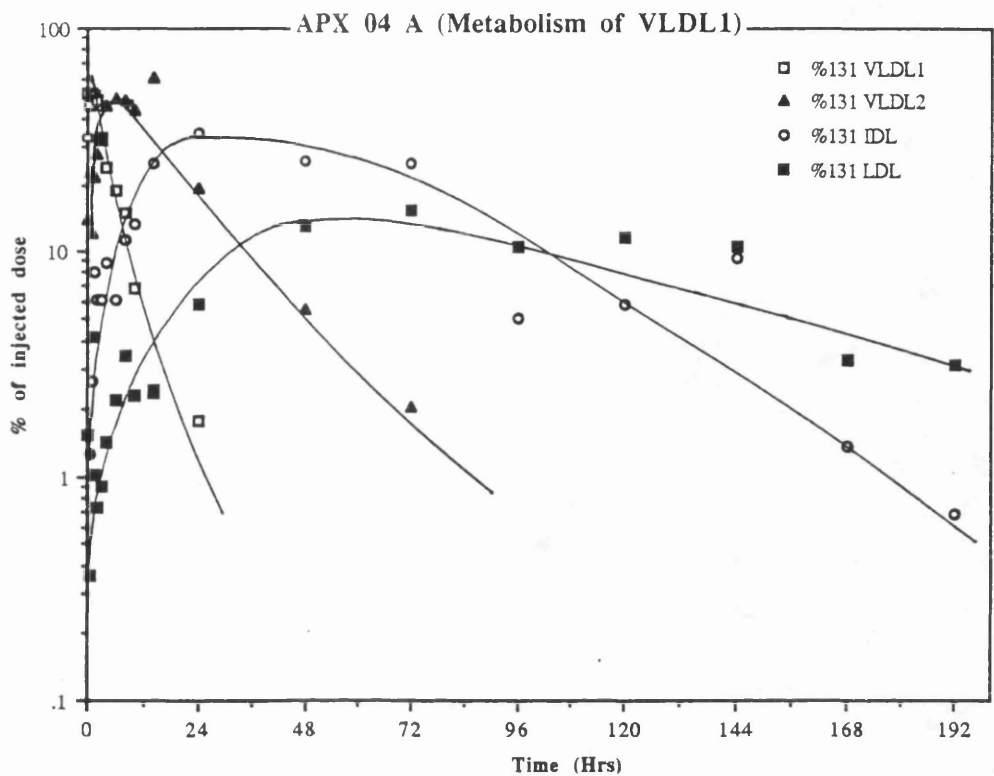


Figure A-79 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{131}$ I]-VLDL<sub>1</sub>.

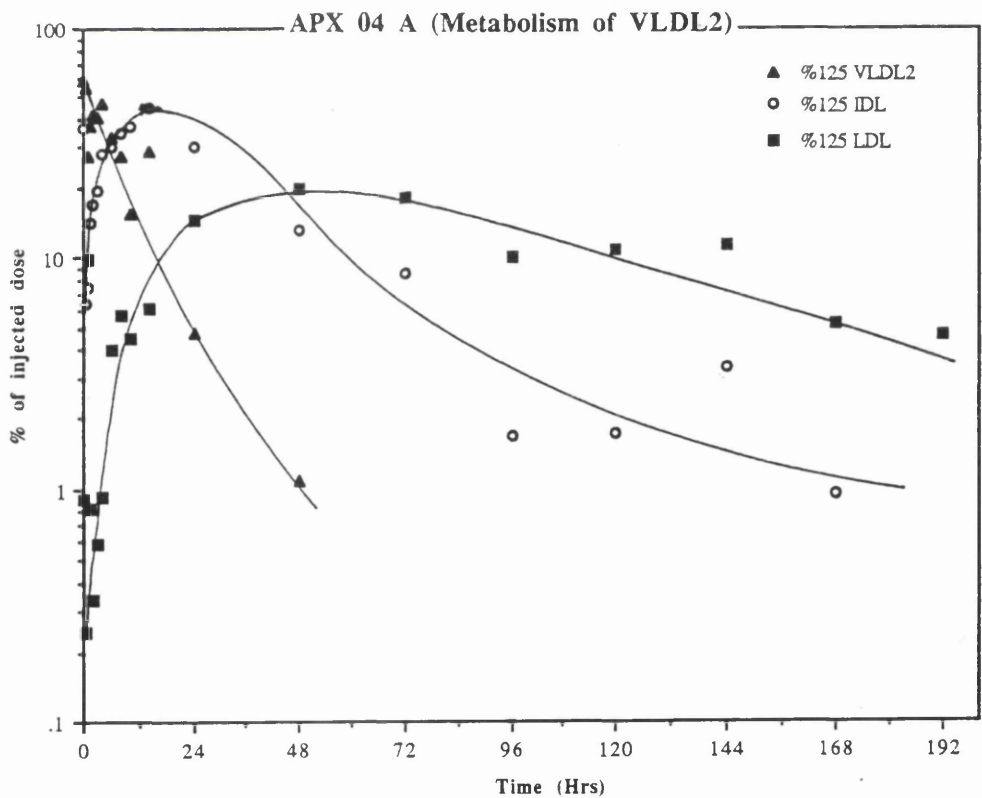


Figure A-80 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [ $^{125}$ I]-VLDL<sub>2</sub>.

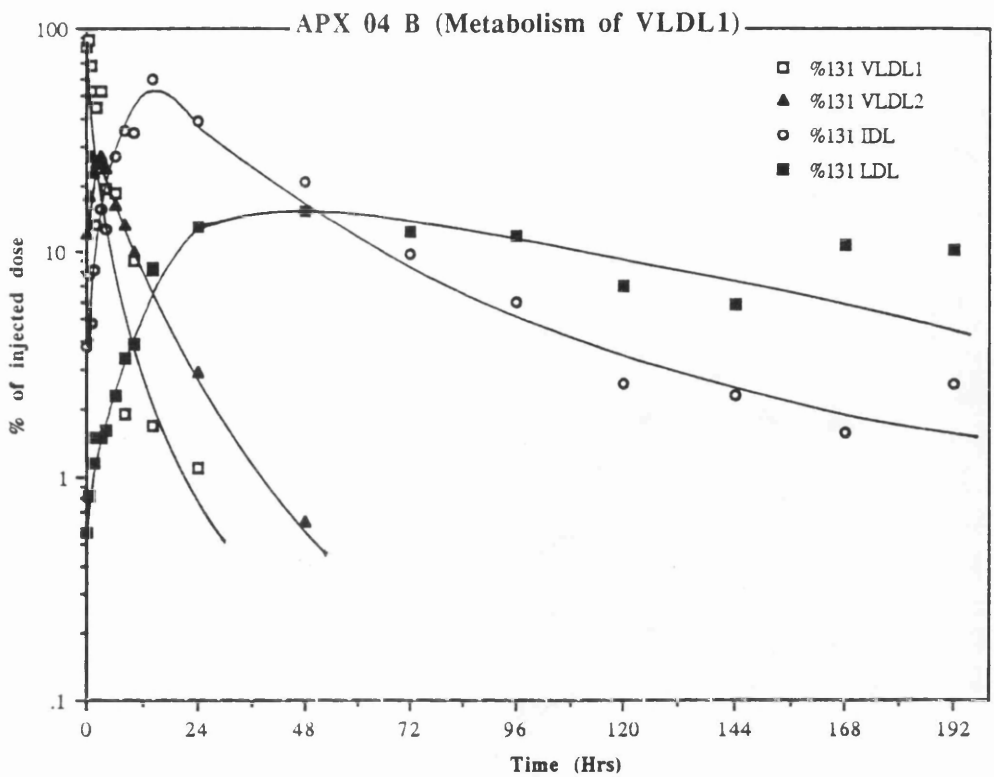


Figure A-81 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

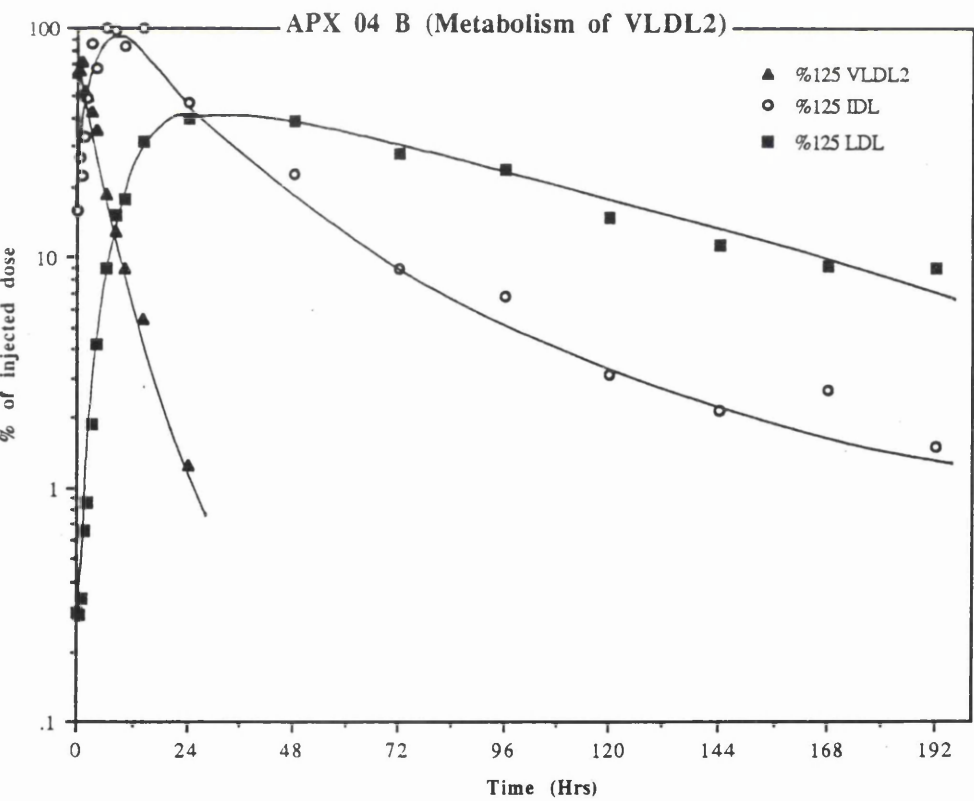


Figure A-82 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

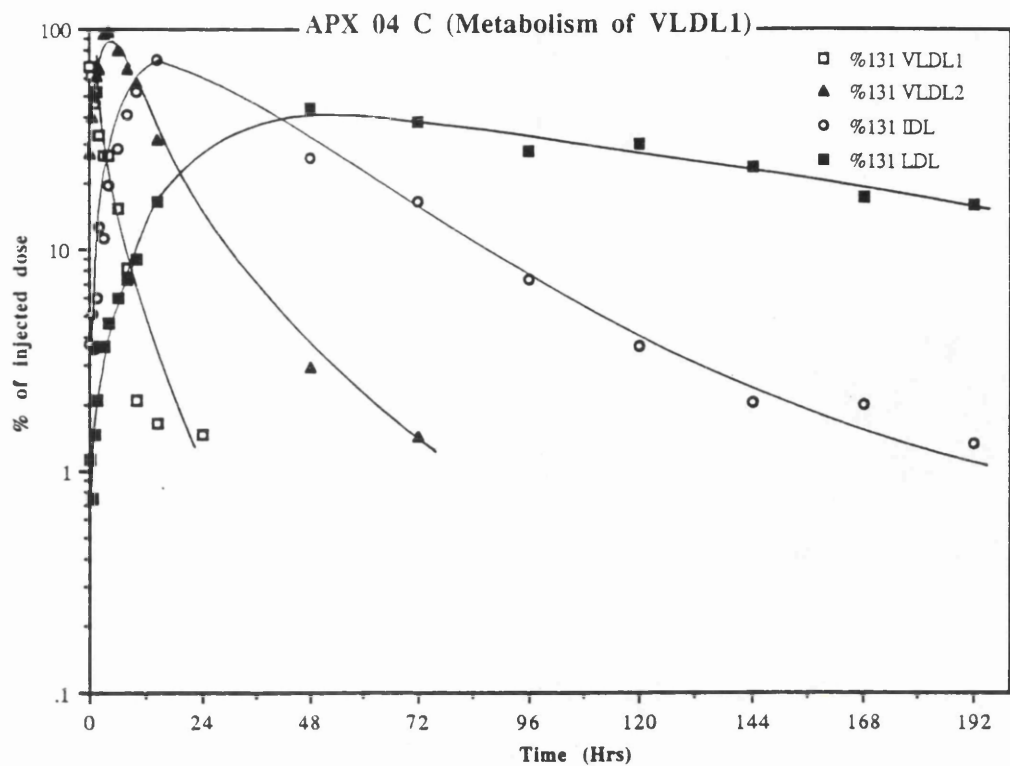


Figure A-83 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

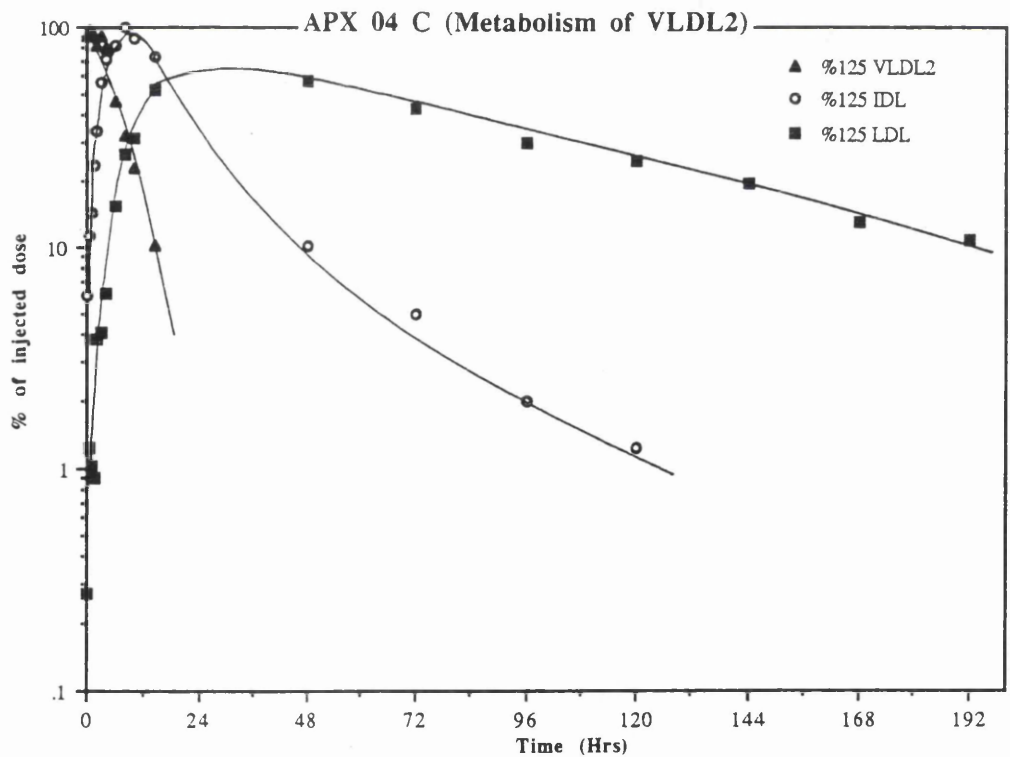


Figure A-84 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

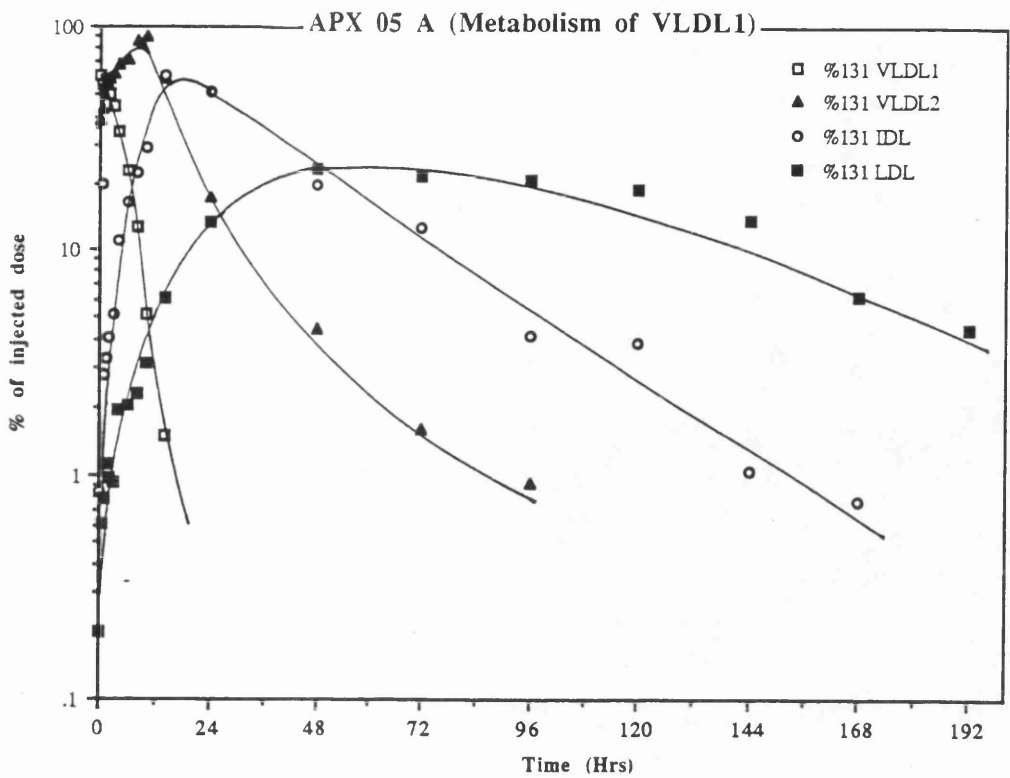


Figure A-85 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

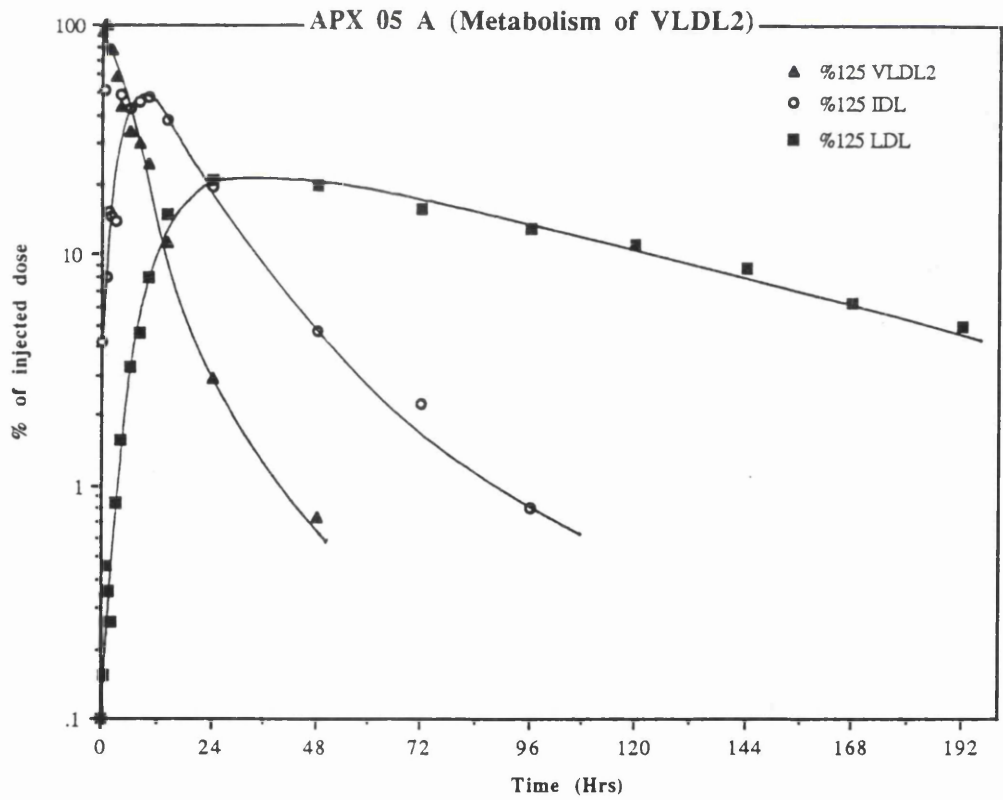


Figure A-86 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

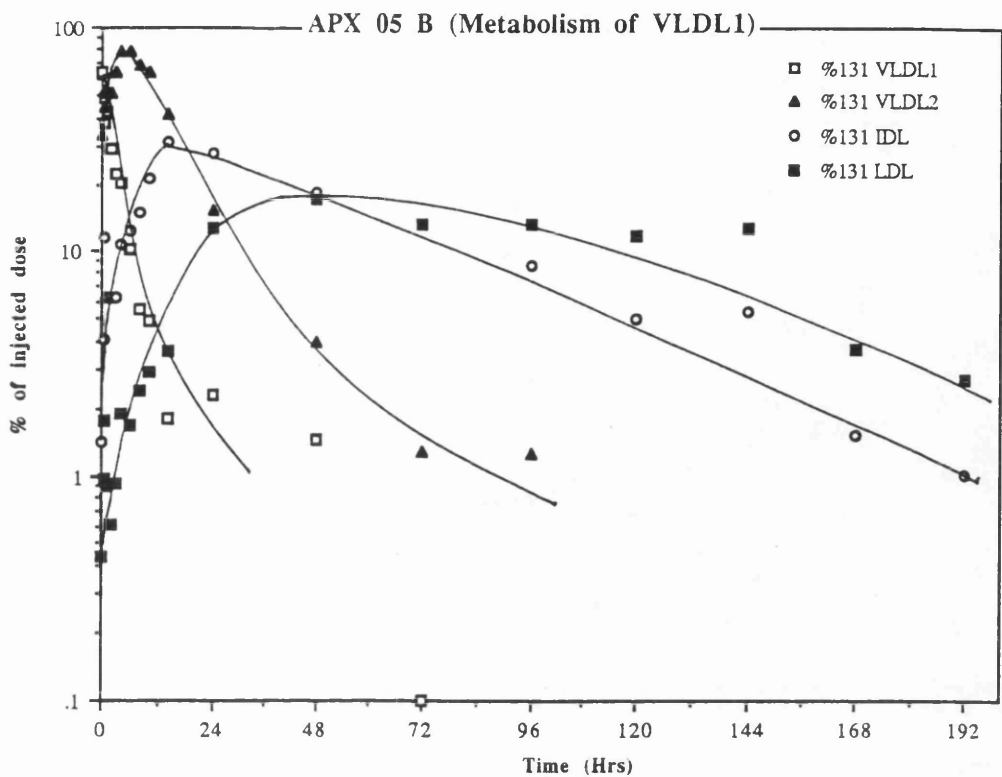


Figure A-87 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

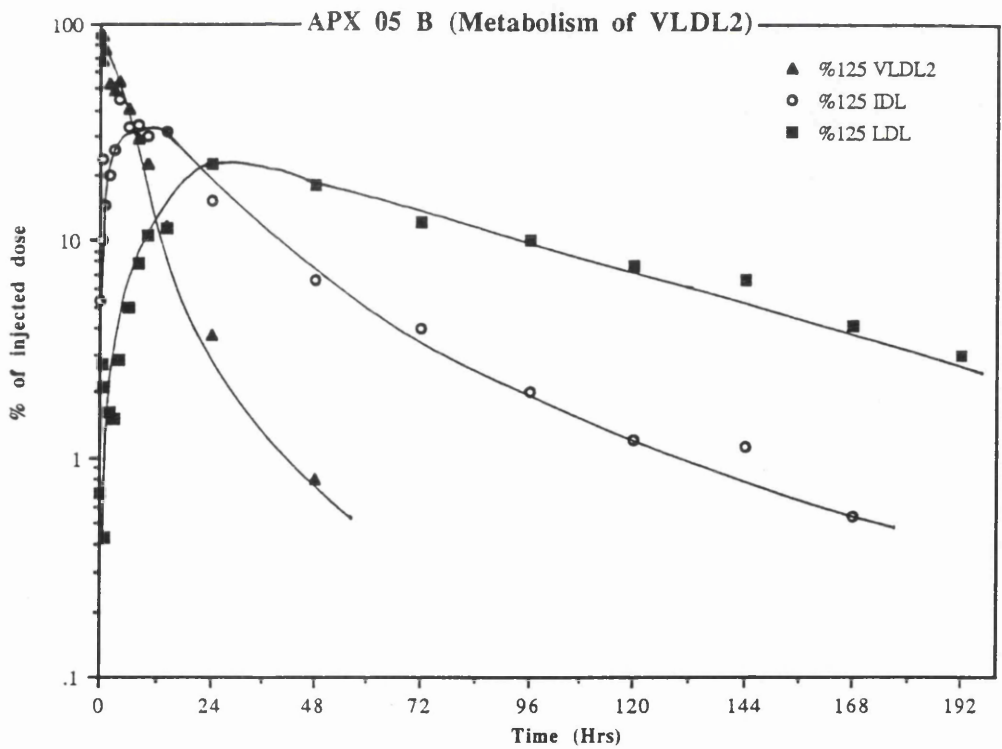
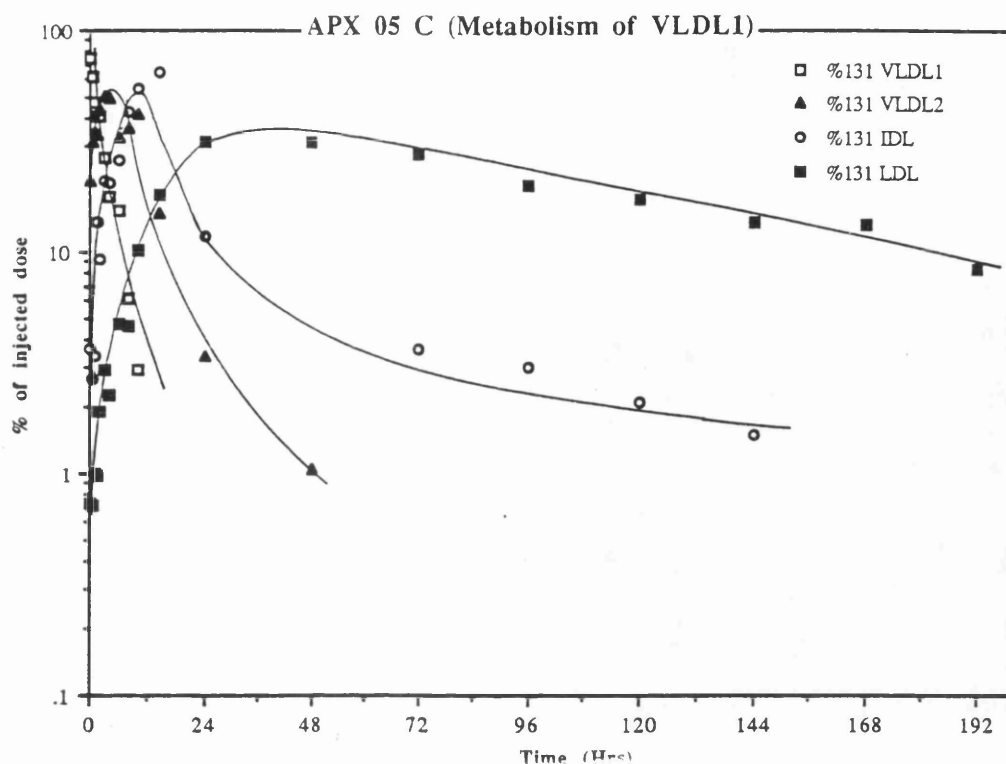
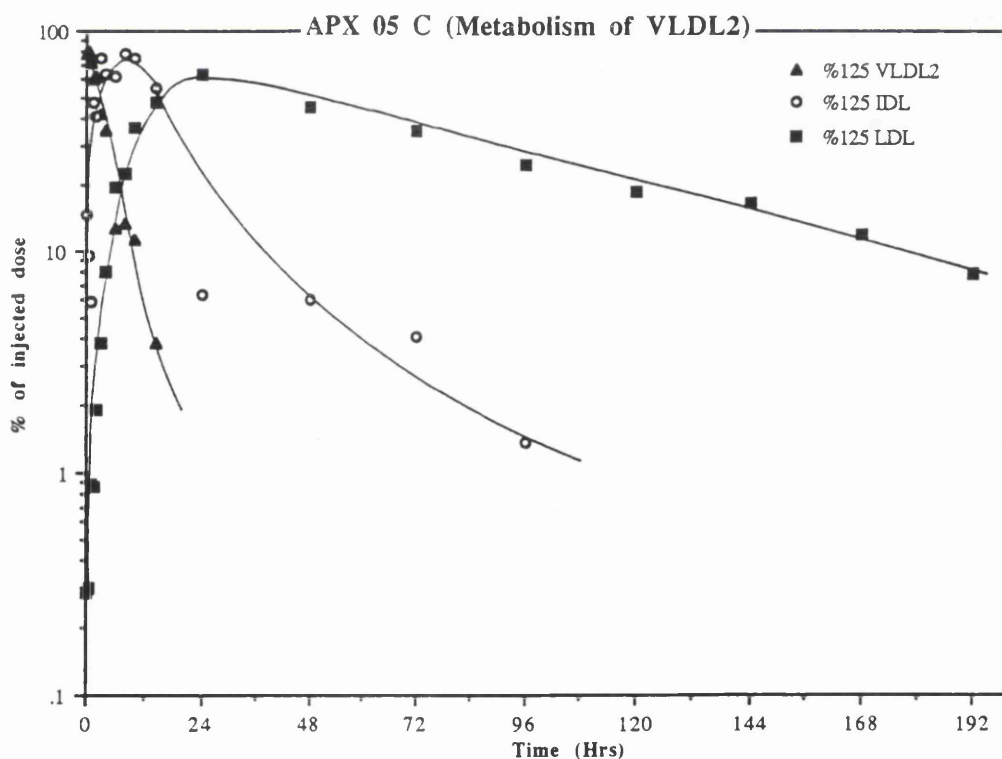


Figure A-88 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.





**Figure A-89** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-90** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

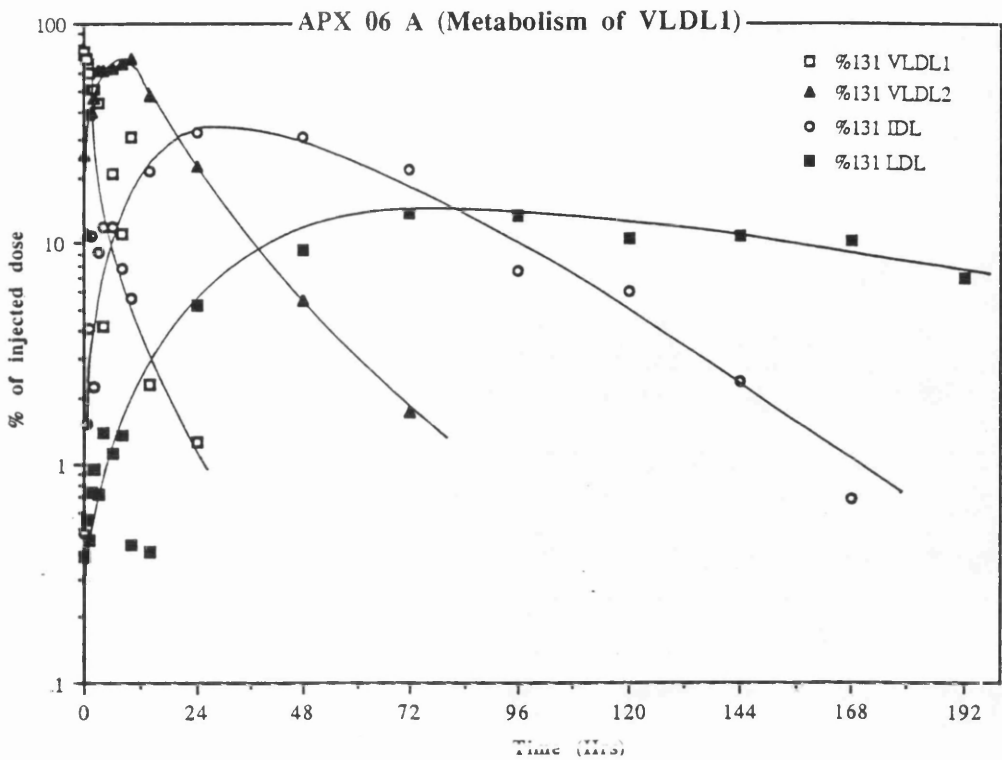


Figure A-91 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

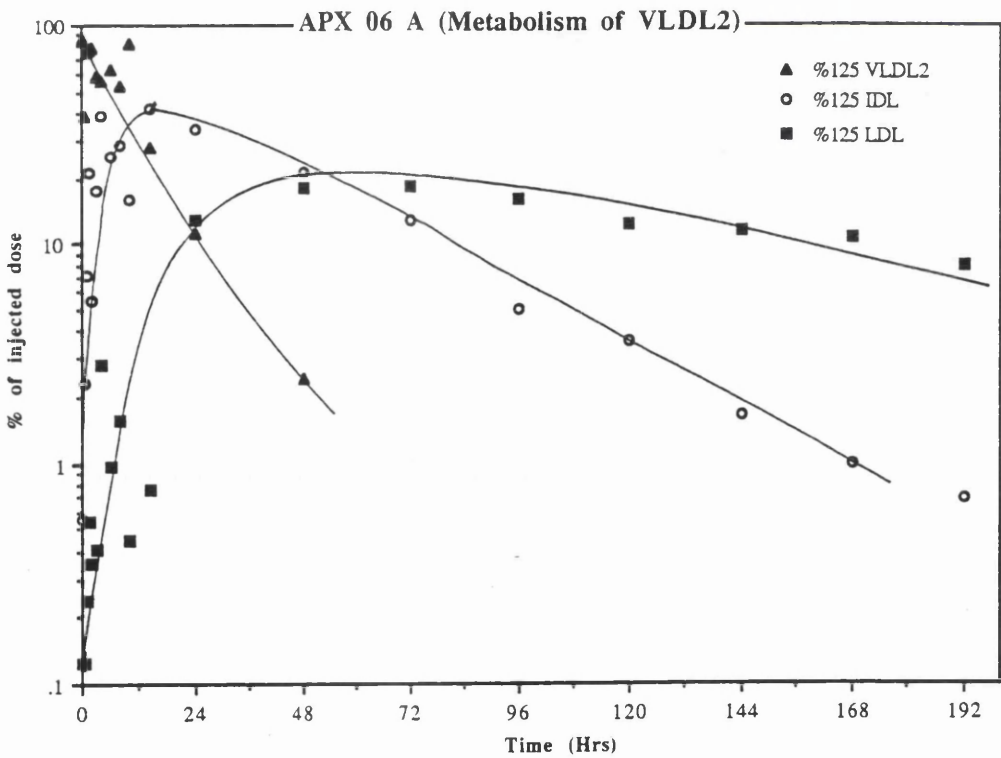


Figure A-92 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

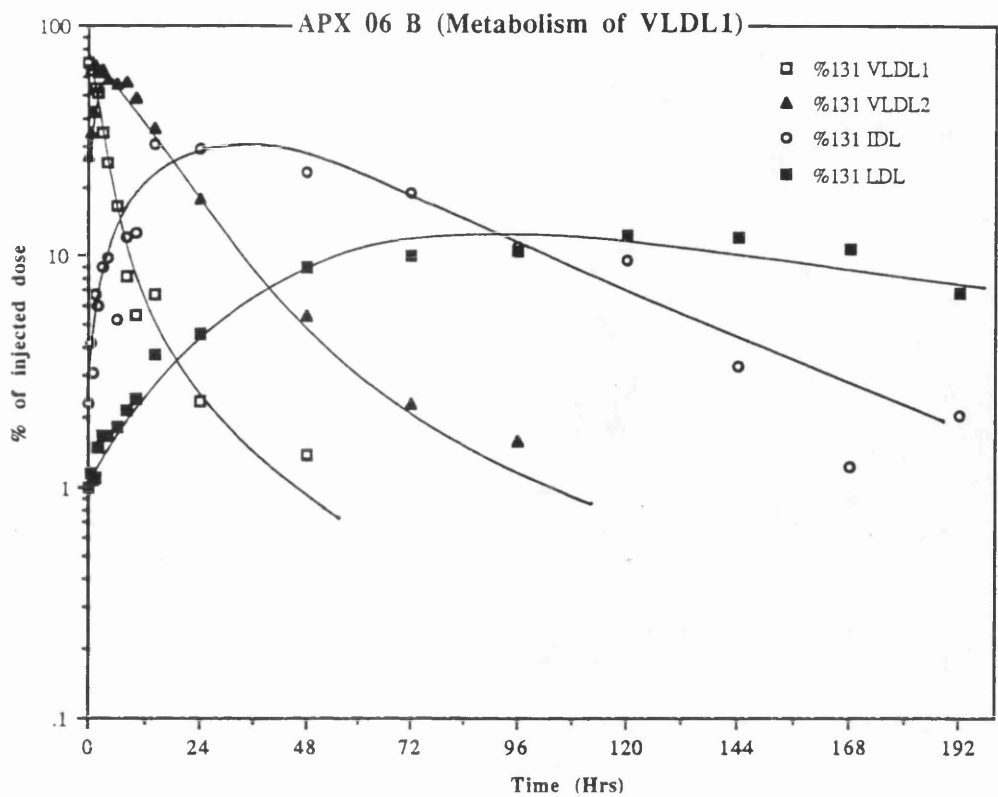


Figure A-93 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

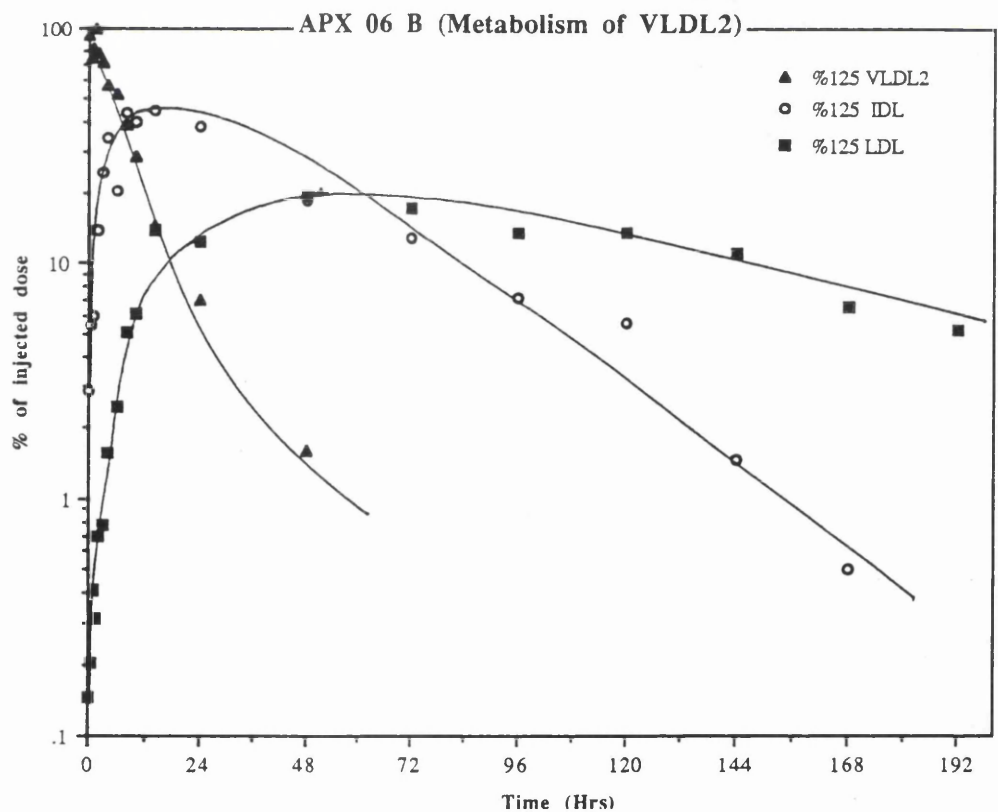


Figure A-94 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

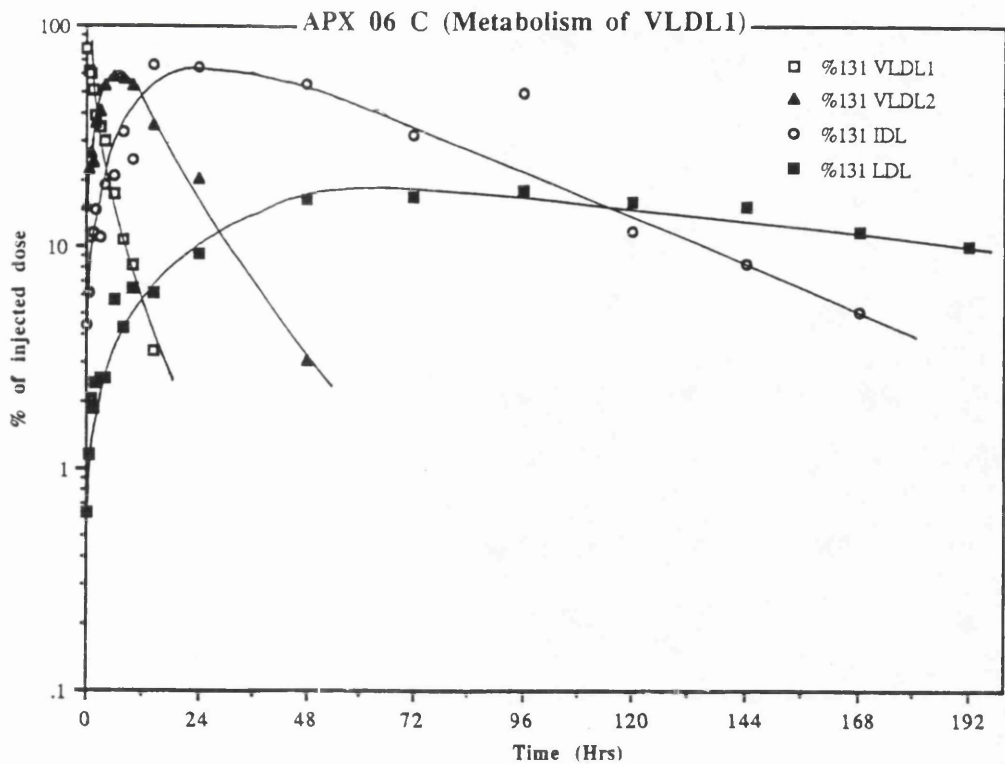


Figure A-95 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

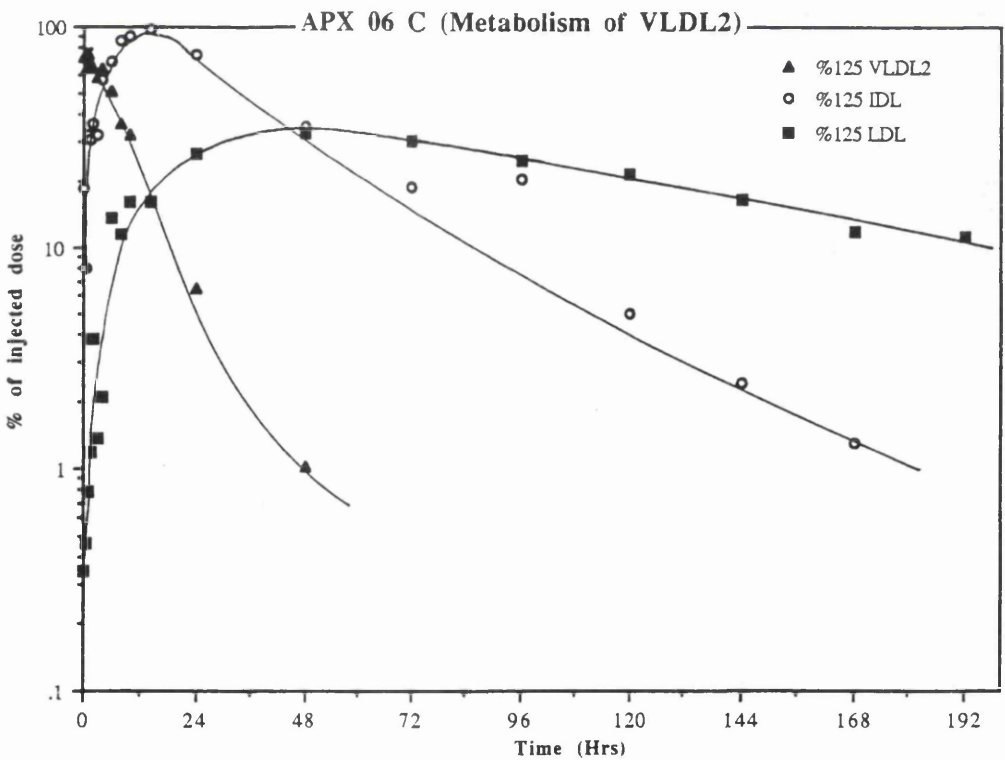


Figure A-96 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

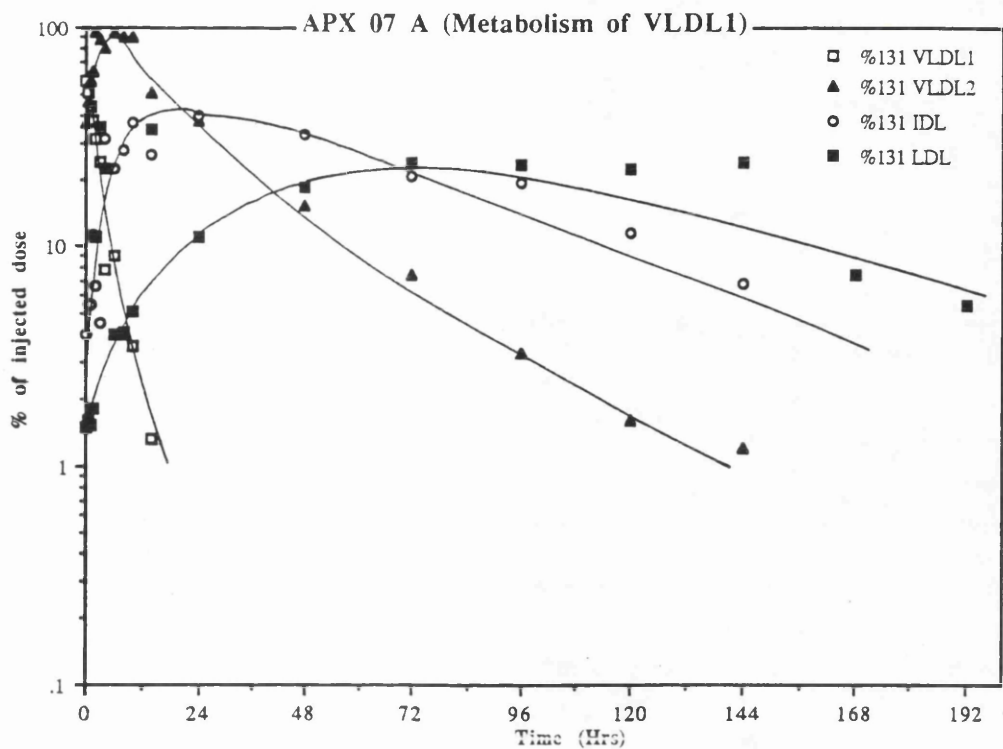


Figure A-97 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.

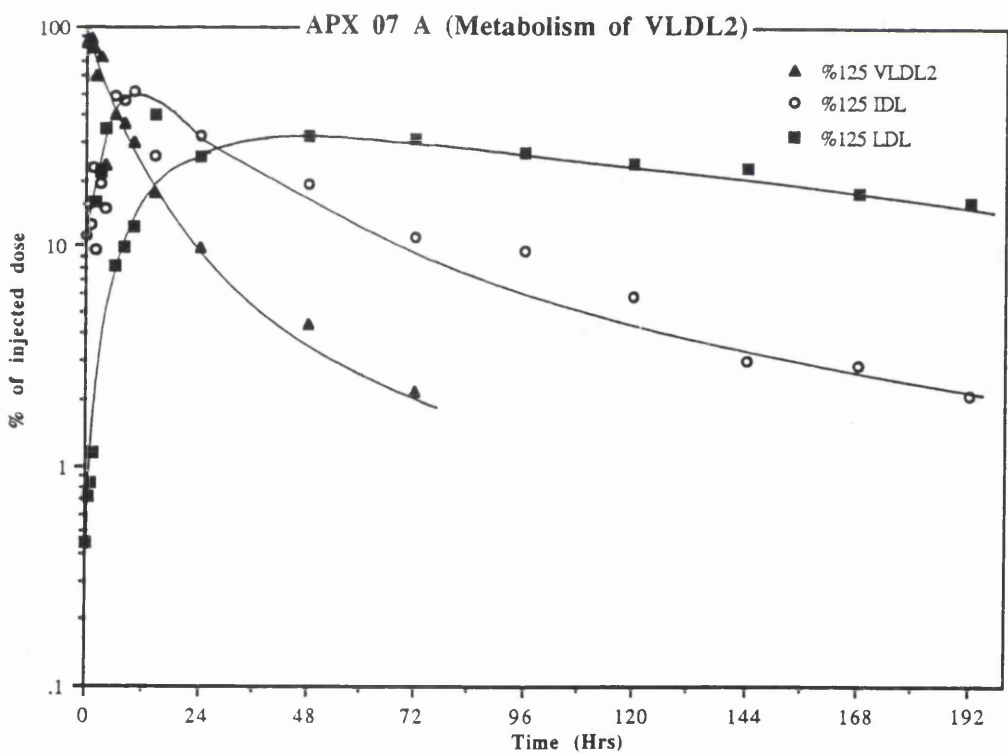
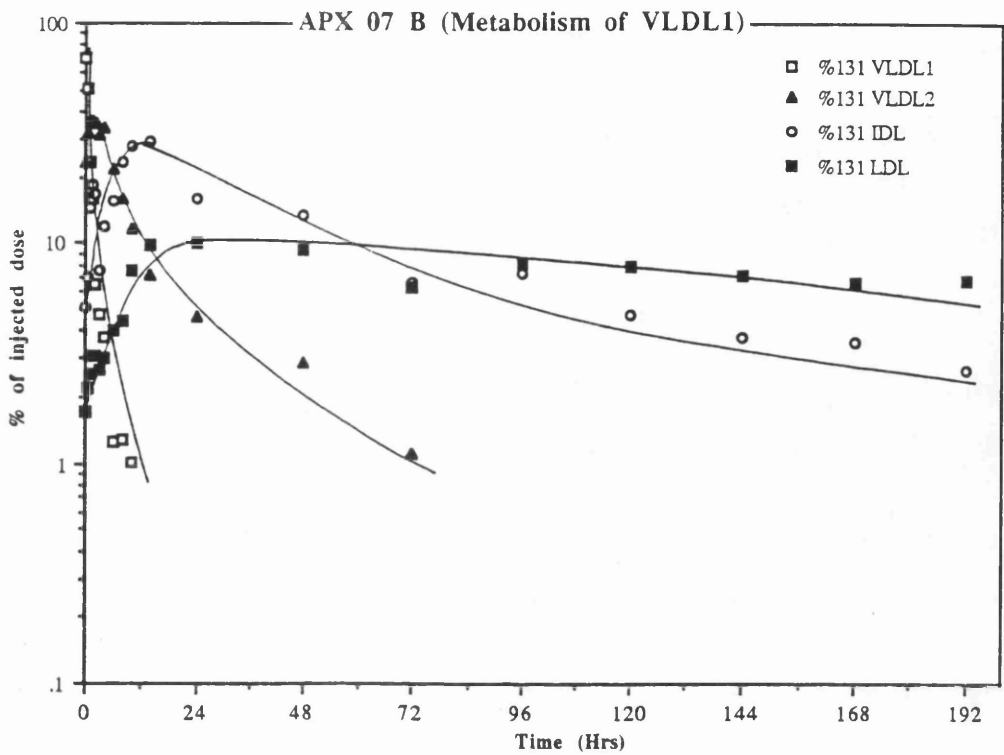
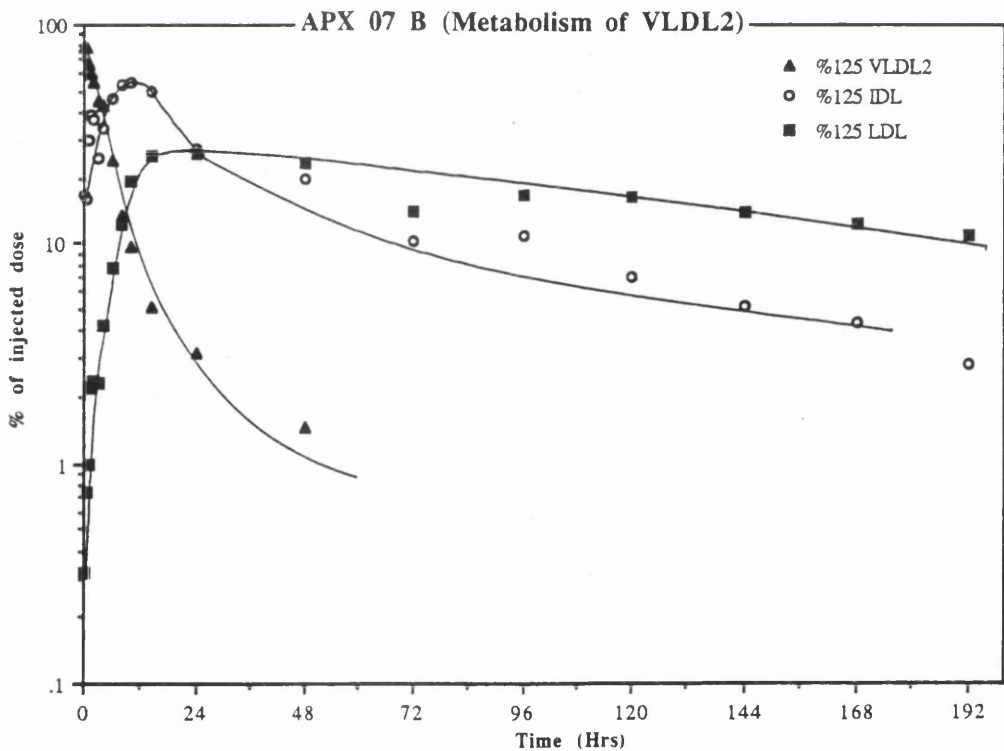


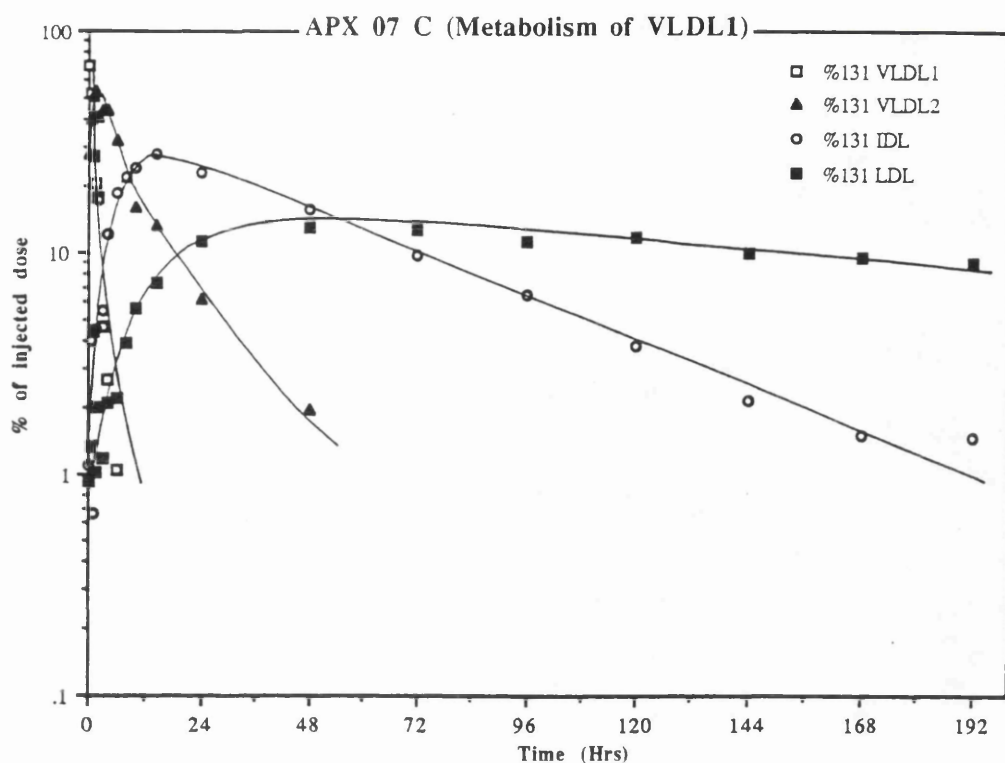
Figure A-98 Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



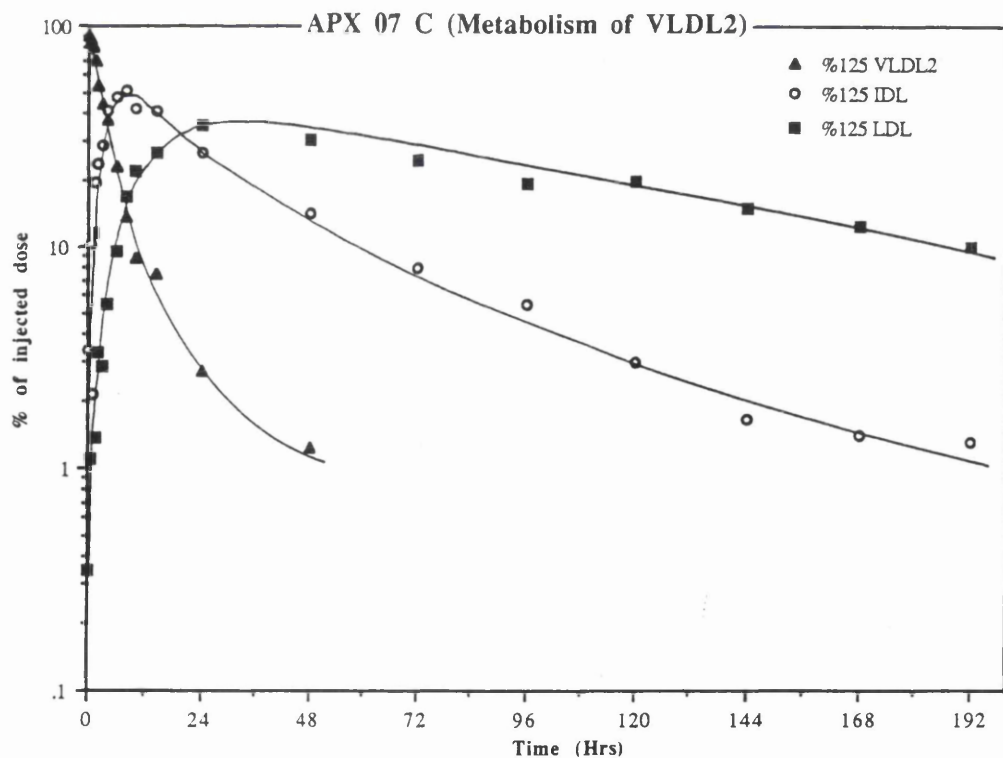
**Figure A-99** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-100** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.



**Figure A-101** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>1</sub>, VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>131</sup>I]-VLDL<sub>1</sub>.



**Figure A-102** Apolipoprotein B radioactivity decay curves associated with VLDL<sub>2</sub>, IDL & LDL after injection of autologous [<sup>125</sup>I]-VLDL<sub>2</sub>.

Table A-11. Computed Masses and Rate Constants at Baseline, on Acipimox, and on Combined Acipimox and Cholestyramine Therapy. VLDL<sub>1</sub>

Before Therapy		L(0,1)*		L(2,1)		L(0,12)		L(1,13)		L(12,13)		M(1)†		M(12)		M(13)		U(13)‡	
Subject																			
APX 01A		1.22		5.96		0.64		7.37		0.02		119		4		116		857	
APX 02A		16.18		9.29		0.73		24.38		0.02		59		2		61		1504	
APX 03A		18.18		16.59		0.75		35.81		0.02		25		1		24		870	
APX 04A		3.30		3.43		0.41		8.03		0.02		67		3		56		452	
APX 05A		3.86		4.80		0.96		6.70		0.23		101		32		132		905	
APX 06A		2.91		6.40		0.58		7.80		0.02		42		2		50		392	
APX 07A		2.93		6.29		0.99		9.67		0.05		38		2		37		352	
Mean		6.94		7.54		0.72		14.25		0.05		64		7		68		556	
SEM		2.67		1.65		0.08		4.29		0.03		13		4		15		151	
On Acipimox																			
APX 01B		3.64		7.80		0.54		12.20		0.02		75		3		70		860	
APX 02B		3.02		4.55		0.23		7.36		0.02		29		3		30		220	
APX 03B		10.51		22.97		1.29		10.82		0.21		4		2		14		136	
APX 04B		0.91		5.26		24.00		31.58		24.00		88		17		17		951	
APX 05B		0.00		9.38		0.38		9.47		0.02		28		2		28		263	
APX 06B		4.18		4.21		0.64		9.30		0.08		43		5		39		364	
APX 07B		24.00		9.75		5.06		48.00		1.00		32		4		22		1100	
Mean		6.61		9.13		4.59		18.38		3.62		43		5		31		762	
SEM		3.17		2.46		3.30		5.84		3.40		11		2		7		154	
On Combination Therapy																			
APX 01C		4.69		4.92		0.59		9.22		0.02		114		5		119		1098	
APX 02C		48.00		6.00		12.75		43.51		0.01		26		0		32		1404	
APX 03C		7.13		21.57		2.50		28.88		0.53		14		3		14		409	
APX 04C		5.35		4.33		0.61		9.86		0.02		34		1		34		330	
APX 05C		2.78		8.75		0.70		11.65		0.02		38		1		38		439	
APX 06C		1.98		4.53		0.51		6.69		0.02		34		2		33		222	
APX 07C		24.00		8.94		0.92		26.45		0.02		29		1		36		965	
Mean		13.42		8.43		2.65		19.47		0.09		41		2		44		695	
SEM		6.42		2.31		1.70		5.20		0.07		12		1		13		172	

\*Rate constants, L(destination, source) are in units, d<sup>-1</sup> † Masses M( ) are in mg. ‡ U( ) represents *de novo* synthesis of apo B into a compartment



Table A-12. Computed Masses and Rate Constants at Baseline, on Acipimox, and on Combined Acipimox and Cholestyramine Therapy. VLDL<sub>2</sub>

Before Therapy		L(4,2)	L(6,2)	L(0,6)	L(0,4)	L(8,4)	L(11,4)	L(9,4)	L(0,5)	L(7,5)	L(10,5)
Subject											
APX 01A		8.28	0.11	0.50	0.72	0.58	0.00	0.40	0.81	2.88	0.01
APX 02A		7.48	0.13	0.73	1.31	1.57	0.01	0.27	2.15	1.81	0.00
APX 03A		24.00	0.34	0.76	0.00	1.32	0.15	1.42	1.23	4.77	0.00
APX 04A		4.41	0.09	0.35	0.47	2.04	0.00	0.26	0.00	3.60	0.00
APX 05A		6.20	0.07	0.60	1.05	1.24	0.00	0.25	0.45	3.72	0.00
APX 06A		6.63	0.20	0.57	0.62	0.73	0.02	0.86	0.00	3.36	0.00
APX 07A		9.53	0.16	0.32	0.21	0.48	0.02	0.74	1.61	3.12	0.00
Mean		9.50	0.16	0.55	0.63	1.14	0.03	0.60	0.89	3.32	0.00
SEM		2.49	0.04	0.07	0.17	0.22	0.02	0.17	0.31	0.34	0.00
On Acipimox											
APX 01B		12.24	0.16	0.57	1.64	2.20	0.00	1.36	0.47	3.49	0.00
APX 02B		9.30	0.02	0.34	1.04	0.13	0.21	1.15	2.33	4.15	0.00
APX 03B		24.00	0.15	0.68	1.59	1.14	0.11	0.72	0.15	9.41	0.00
APX 04B		22.76	0.23	0.79	0.00	5.37	0.00	0.42	0.00	4.34	0.00
APX 05B		14.17	0.13	0.33	0.00	1.82	0.00	0.31	0.13	4.78	0.00
APX 06B		3.60	0.04	0.34	0.53	2.08	0.03	0.00	0.84	2.81	0.00
APX 07B		14.82	0.21	0.47	0.76	1.75	0.37	2.01	5.29	4.51	0.00
Mean		11.13	0.13	0.50	0.79	2.07	0.10	0.85	1.32	4.78	0.00
SEM		2.77	0.03	0.07	0.26	0.61	0.05	0.26	0.73	0.81	0.00
On Combination Therapy											
APX 01C		8.41	0.14	0.55	1.16	1.98	0.00	0.00	0.27	2.58	0.00
APX 02C		4.33	0.02	0.58	2.04	1.24	0.24	0.59	0.30	3.52	0.00
APX 03C		24.00	0.14	0.40	0.00	3.11	0.50	0.22	3.19	5.14	0.00
APX 04C		8.49	0.02	0.50	1.11	1.77	0.69	0.23	0.01	3.64	0.00
APX 05C		19.73	1.33	1.45	1.49	2.39	0.00	0.46	0.00	8.02	0.00
APX 06C		17.52	0.41	0.68	0.90	1.41	0.00	1.14	0.17	4.26	0.00
APX 07C		10.53	0.16	0.54	0.20	2.96	0.10	0.14	2.04	5.03	0.00
Mean		13.29	0.32	0.67	0.99	2.12	0.22	0.40	0.85	4.60	0.00
SEM		2.71	0.18	0.13	0.27	0.28	0.11	0.14	0.47	0.66	0.00

Table A-13.Computed Masses and Rate Constants at Baseline, on Acipimox, and on Combined Acipimox and Cholestyramine Therapy.. VLDL<sub>2</sub> (contd)

Before Therapy Subject					
	M(2)	M(4)	M(6)	M(5)	U(5)
APX 01A	84	413	18	169	626
APX 02A	72	169	12	234	928
APX 03A	17	138	8	82	492
APX 04A	51	81	13	178	640
APX 05A	78	190	9	282	1178
APX 06A	39	116	14	83	279
APX 07A	25	164	12	154	728
Mean	52	182	12	169	696
SEM	10	41	1	28	110
On Acipimox					
APX 01B	47	111	13	198	784
APX 02B	14	52	1	190	1231
APX 03B	4	28	1	79	756
APX 04B	20	79	6	191	830
APX 05B	19	124	7	190	932
APX 06B	50	68	6	132	481
APX 07B	20	62	9	92	901
Mean	25	75	6	153	845
SEM	7	13	2	19	85
On Combination Therapy					
APX 01C	66	176	17	219	624
APX 02C	36	38	1	171	653
APX 03C	13	79	4	233	1941
APX 04C	17	39	1	124	452
APX 05C	16	72	14	117	938
APX 06C	9	43	5	132	584
APX 07C	24	74	7	112	792
Mean	26	74	7	158	855
SEM	7	18	2	19	190

Table A-14. Computed Masses and Rate Constants at Baseline, on Acipimox, and on Combined Acipimox and Cholestyramine Therapy. IDL

Before Therapy								
Subject	L(0,8)	L(11,8)	L(0,9)	L(0,7)	L(10,7)	M(8)	M(9)	M(7)
APX 01A	0.20	0.91	0.34	0.38	1.37	213	488	277
APX 02A	0.08	0.50	0.14	0.58	1.19	457	327	239
APX 03A	0.02	2.00	0.50	0.00	1.97	91	391	197
APX 04A	0.41	0.55	0.23	0.42	0.99	173	90	453
APX 05A	0.13	1.09	0.30	0.27	1.43	192	161	616
APX 06A	0.00	0.89	0.48	0.25	1.03	95	207	217
APX 07A	0.00	1.05	0.43	0.00	1.50	75	280	321
Mean	0.12	1.00	0.35	0.27	1.35	185	278	331
SEM	0.06	0.19	0.05	0.08	0.13	50	52	57
On Acipimox								
APX 01B	0.51	0.69	0.51	0.21	1.67	202	292	367
APX 02B	0.52	0.48	0.16	0.38	0.79	7	374	672
APX 03B	0.65	0.34	0.19	0.94	1.44	33	109	312
APX 04B	0.43	0.45	0.19	0.99	0.36	480	179	614
APX 05B	0.33	0.96	0.26	1.09	1.24	174	148	389
APX 06B	0.25	0.35	0.00	0.00	1.28	235	0	291
APX 07B	1.31	0.59	0.25	0.20	0.98	57	490	351
Mean	0.57	0.55	0.22	0.54	1.11	170	227	428
SEM	0.13	0.08	0.06	0.17	0.17	62	63	57
On Combination Therapy								
APX 01C	0.53	0.28	0.41	0.00	1.26	433	0	447
APX 02C	0.24	0.24	0.14	0.48	0.72	96	154	501
APX 03C	0.79	0.40	0.16	0.57	2.02	205	109	463
APX 04C	0.17	0.66	0.17	0.26	1.21	83	53	307
APX 05C	0.25	1.85	0.36	1.37	1.90	82	92	286
APX 06C	0.30	0.72	0.23	0.11	0.79	60	218	618
APX 07C	0.33	0.32	0.11	0.00	1.79	339	89	315
Mean	0.37	0.64	0.23	0.40	1.38	185	102	420
SEM	0.08	0.21	0.04	0.18	0.20	56	26	46

Table A-15. Computed Masses and Rate Constants at Baseline, on Acipimox, and on Combined Acipimox and Cholestyramine Therapy. LDL

*Before Therapy*

Subject	L(0,11)	L(0,10)	M(11)	M(10)
APX 01A	0.22	0.24	899	1570
APX 02A	0.19	1.53	1192	186
APX 03A	0.18	0.21	1107	1814
APX 04A	0.18	0.29	519	1543
APX 05A	0.20	0.46	1025	1909
APX 06A	0.17	0.24	509	913
APX 07A	0.15	0.19	539	2600
Mean	0.18	0.45	827	1505
SEM	0.01	0.18	113	291

*On Acipimox*

APX 01B	0.17	0.30	838	2053
APX 02B	0.06	0.36	228	1475
APX 03B	0.13	0.36	106	1256
APX 04B	0.35	0.15	612	1452
APX 05B	0.29	0.31	579	1560
APX 06B	0.23	0.39	373	954
APX 07B	0.07	0.19	787	1830
Mean	0.19	0.29	503	1511
SEM	0.04	0.03	105	136

*On Combination Therapy*

APX 01C	0.16	0.32	746	1781
APX 02C	0.14	0.35	227	1034
APX 03C	0.22	0.44	557	2135
APX 04C	0.15	0.35	532	1062
APX 05C	0.22	0.30	698	1798
APX 06C	0.14	0.32	301	1508
APX 07C	0.18	0.31	641	1816
Mean	0.17	0.34	529	1590
SEM	0.01	0.02	74	156

*Appendix 5*

Figure 103 Native LDL

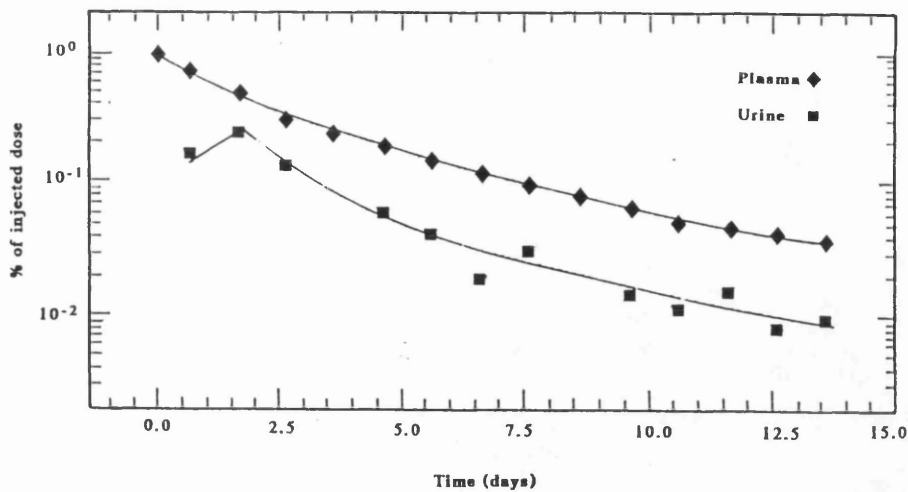


Figure 104 Cyclohexanedione Modified LDL

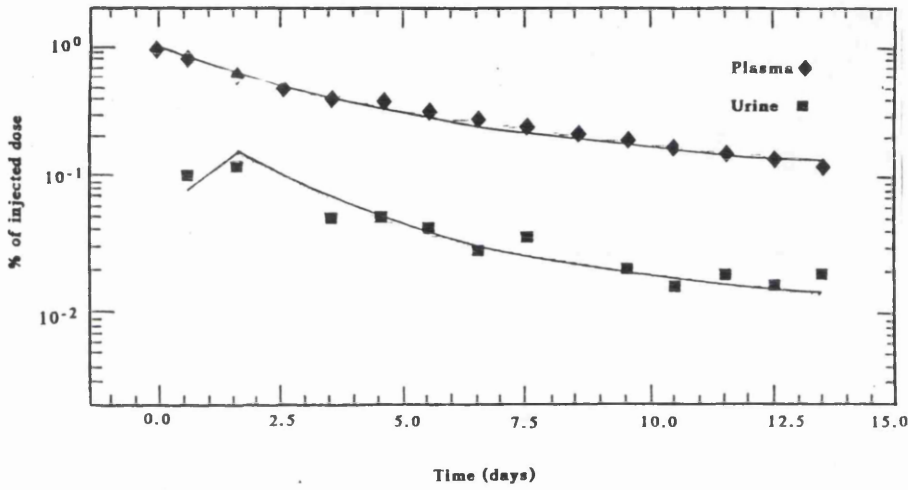
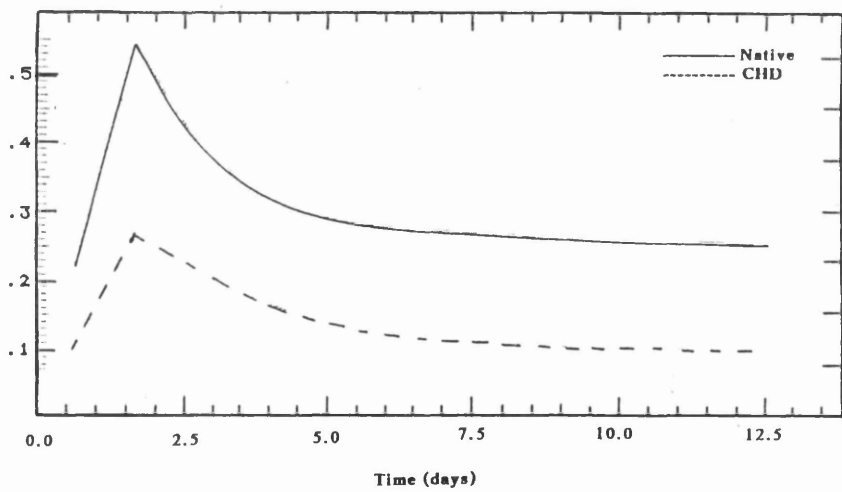


Figure 105 U/P Ratios



Figures A-103-105. CIP 01A. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure 106 Native LDL

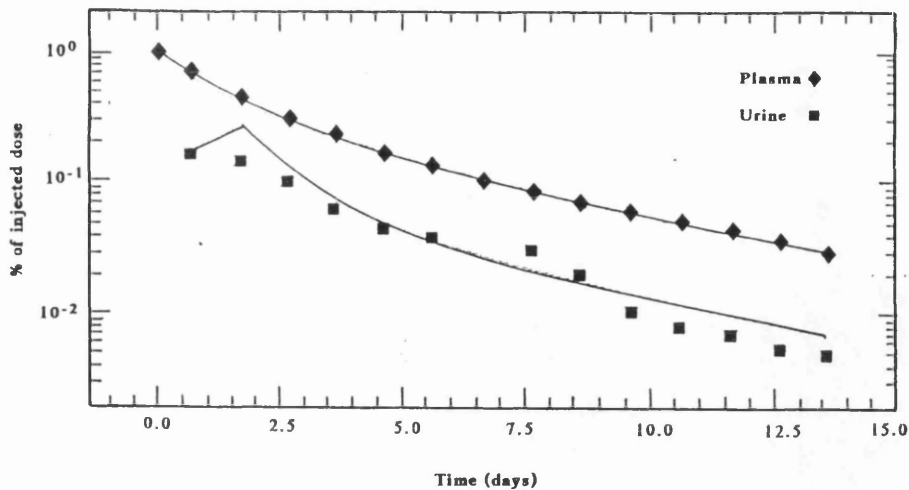


Figure 107 Cyclohexanedione Modified LDL

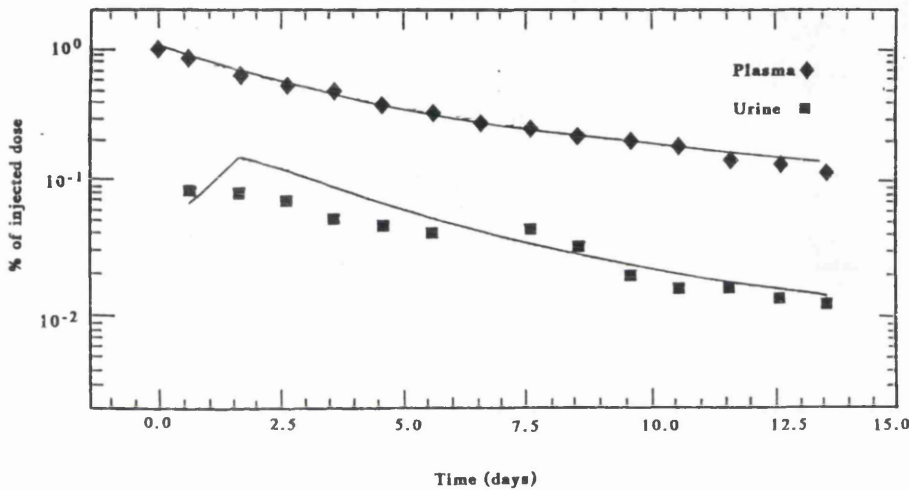
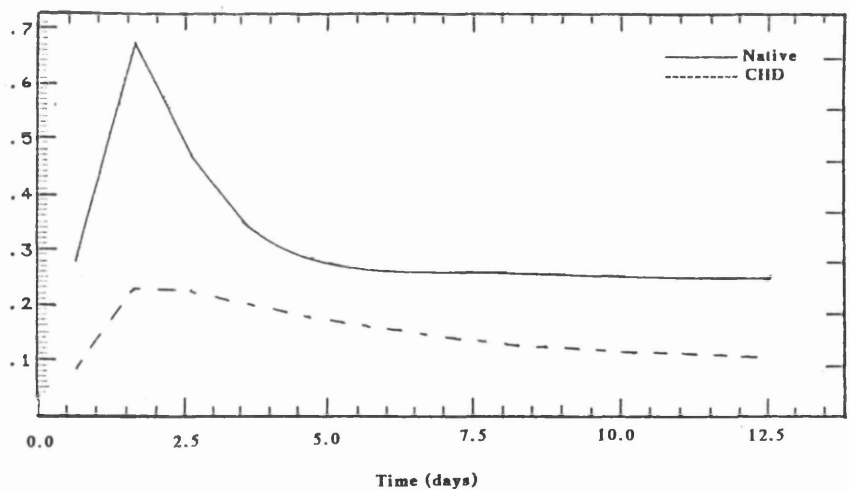


Figure 108 U/P Ratios



Figures A-106-108. CIP 01B. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 109 Native LDL

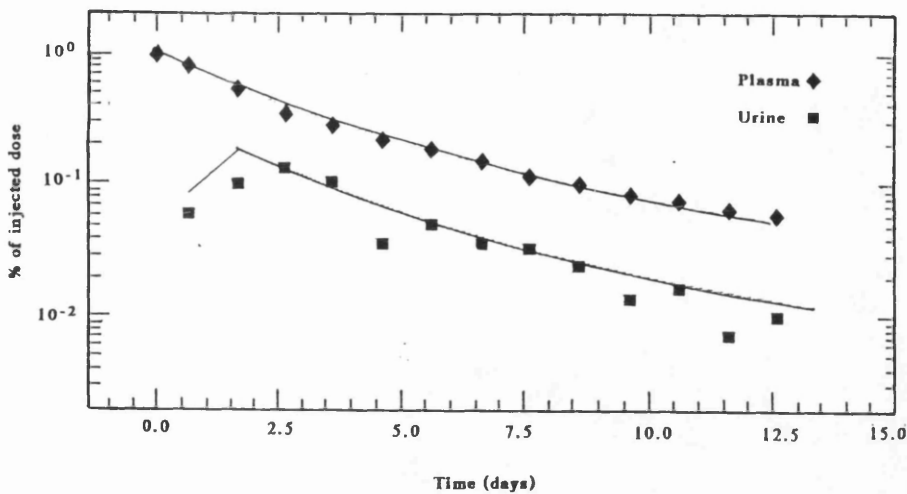


Figure 110 Cyclohexanedione Modified LDL

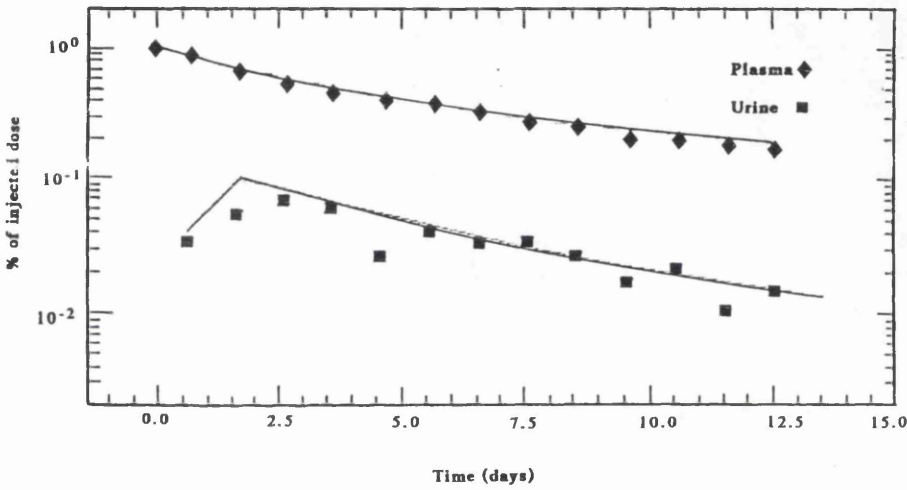
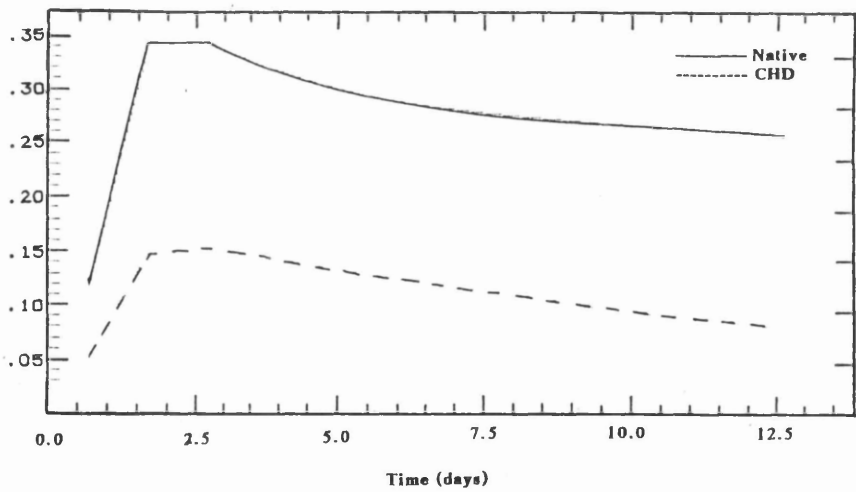


Figure 111 U/P Ratios



Figures A-109-111. CIP 02A. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.



Figure 112 Native LDL

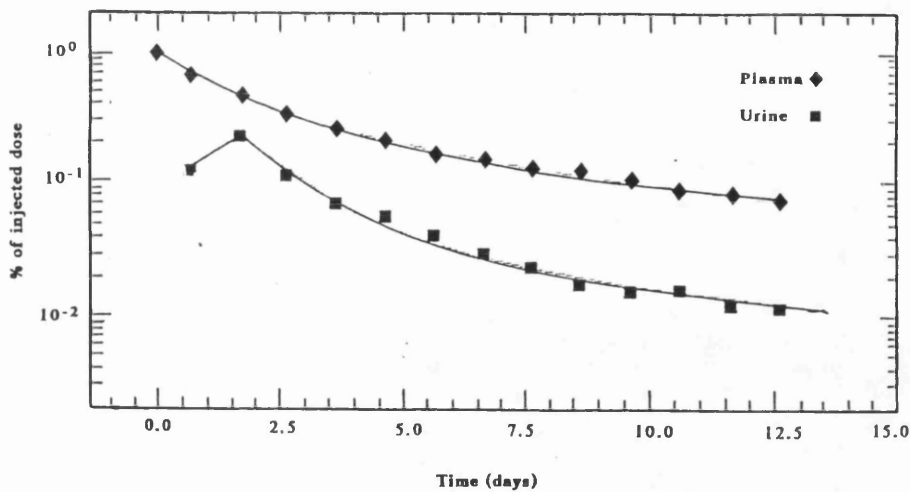


Figure 113 Cyclohexanedione Modified LDL

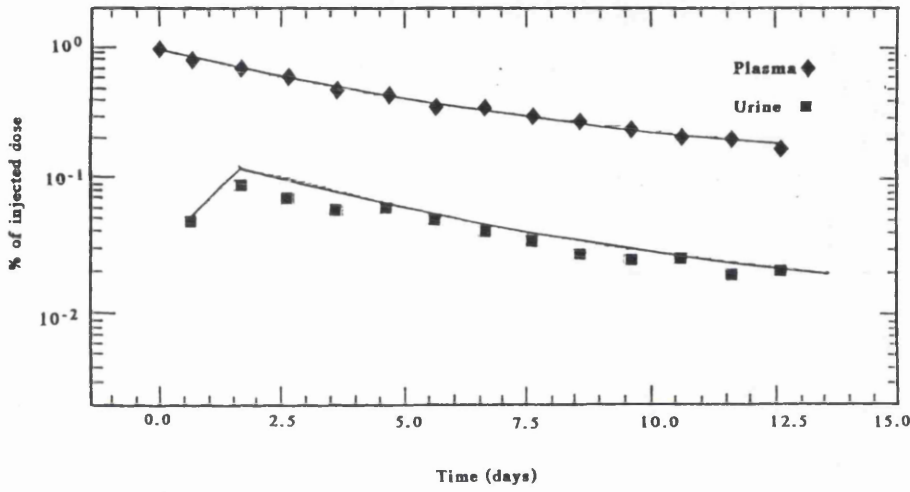
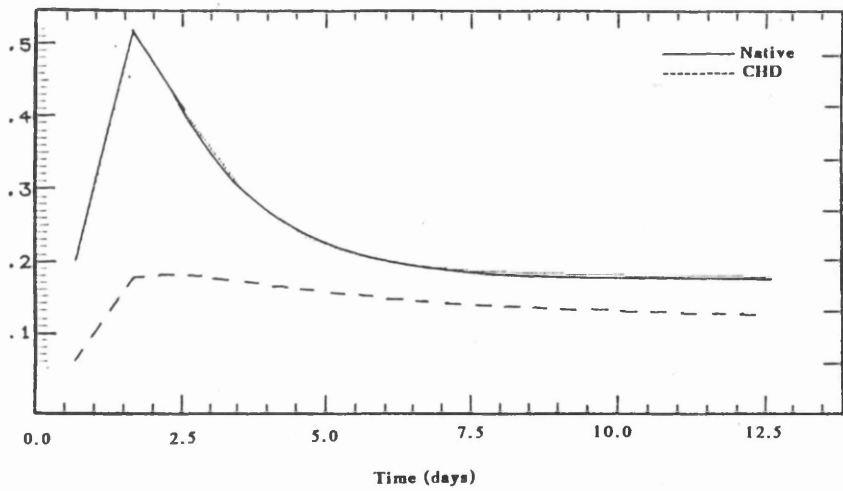


Figure 114 U/P Ratios



Figures A-112-114. CIP 02B. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 115 Native LDL

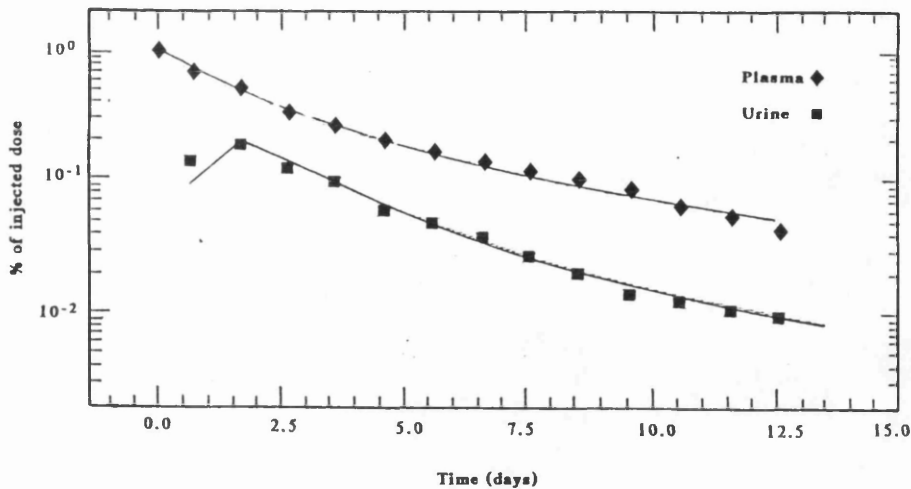


Figure 116 Cyclohexanedione Modified LDL

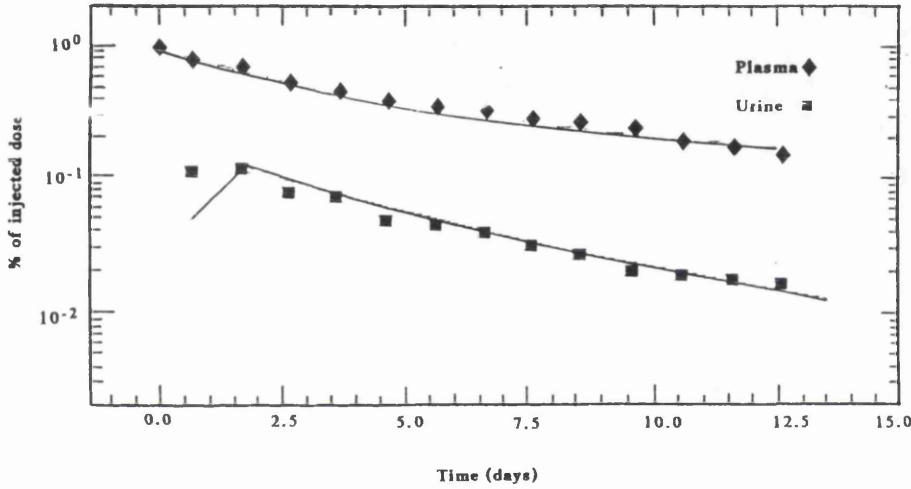
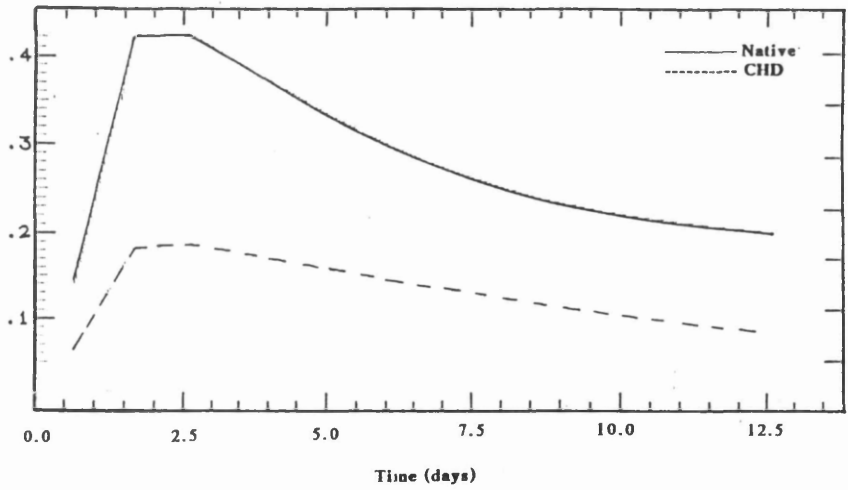


Figure 117 U/P Ratios



Figures A-115-117. CIP 03A. Plasma and urinary radioactivity decay curves following injection of [ $^{125}\text{I}$ ]-native apo-LDL and [ $^{131}\text{I}$ ]-cyclohexanedione modified apo-LDL.

Figure118 Native LDL

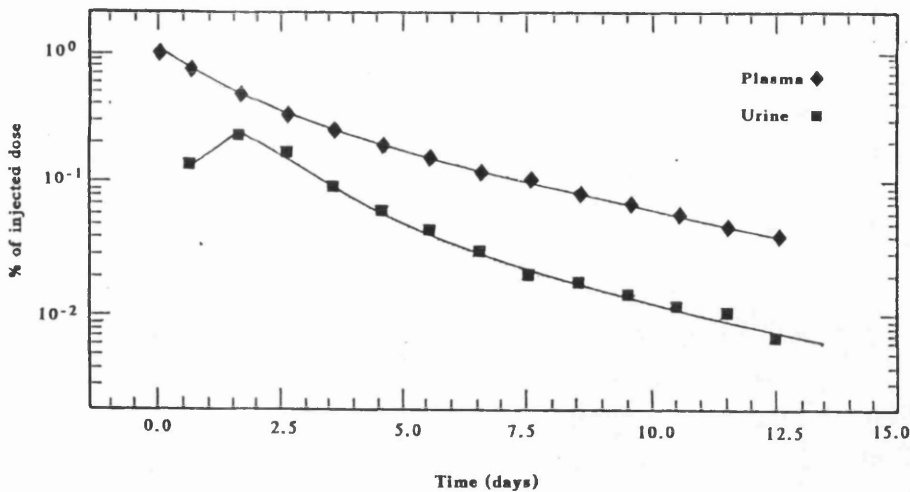


Figure119 Cyclohexanedione Modified LDL

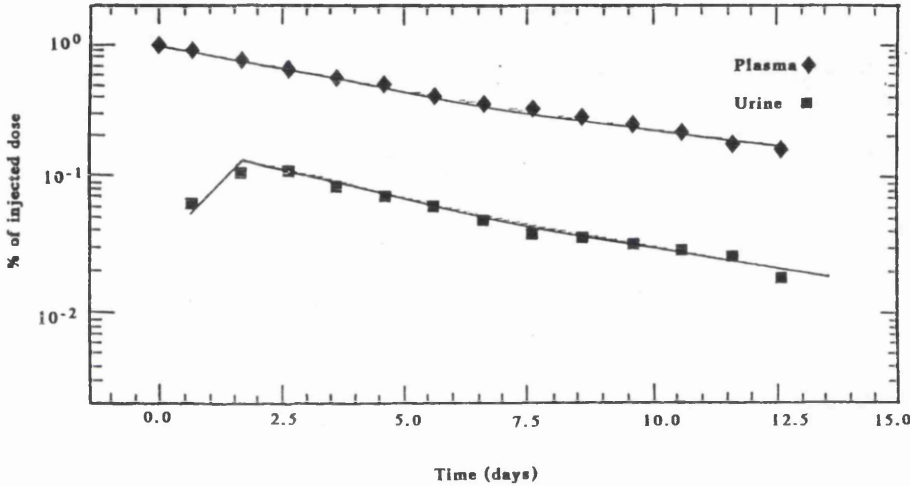
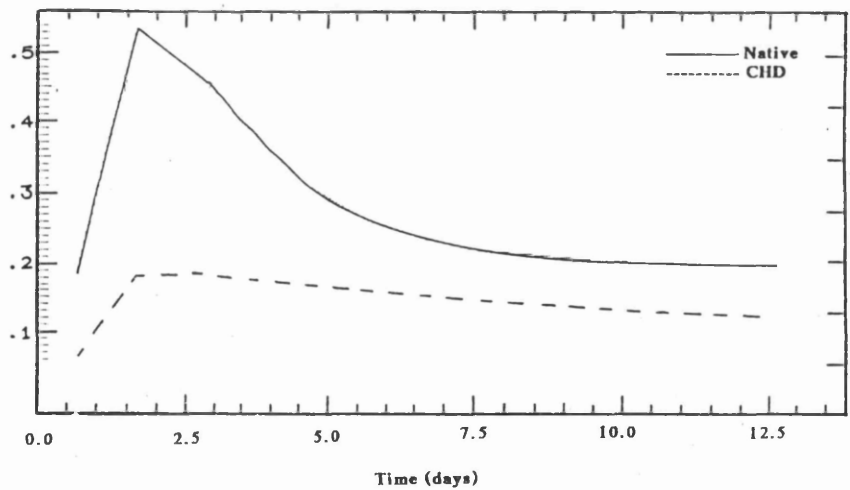


Figure120 U/P Ratios



Figures A-118-120. CIP 03B. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure121 Native LDL

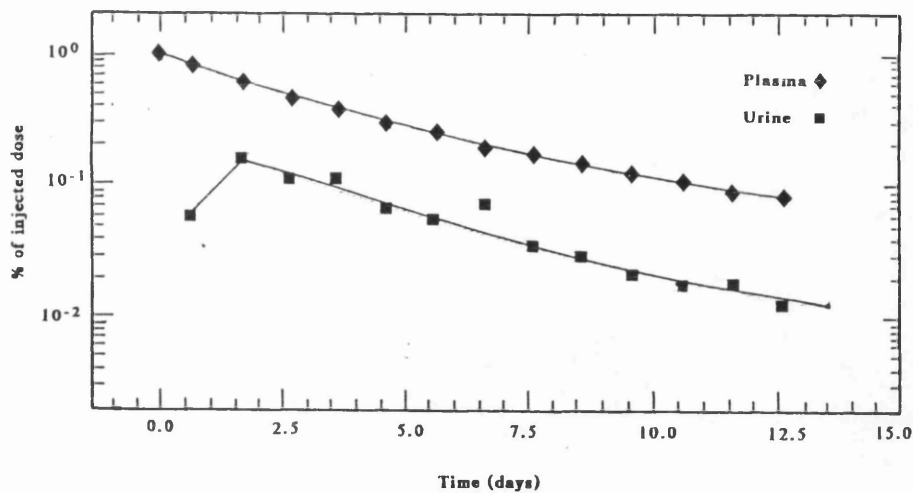


Figure122 Cyclohexanedione Modified LDL

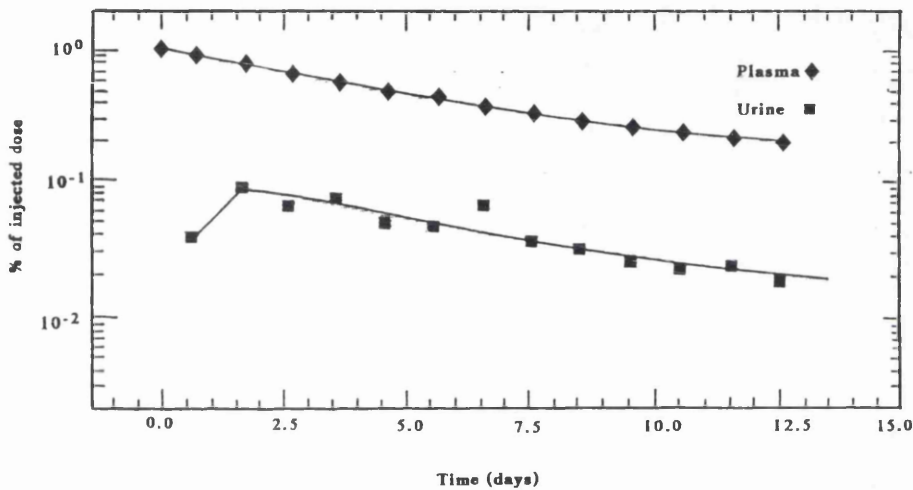
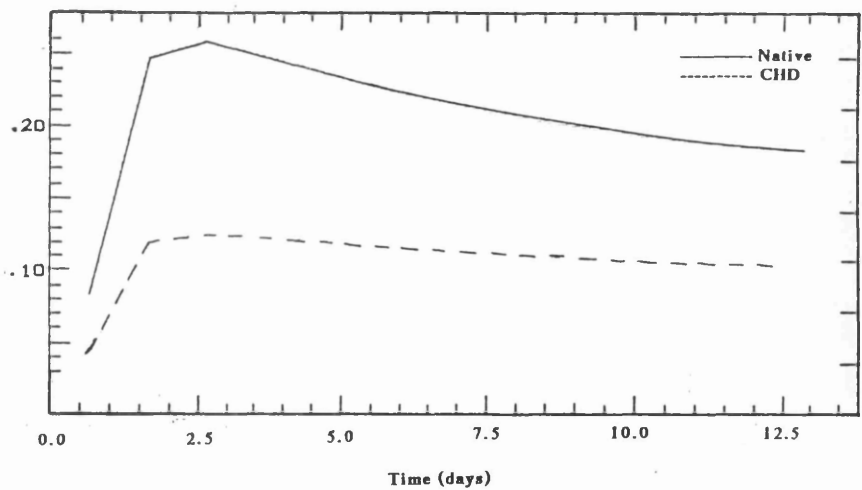


Figure123 U/P Ratios



Figures A-121-123. CIP 04A. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure 127 Native LDL

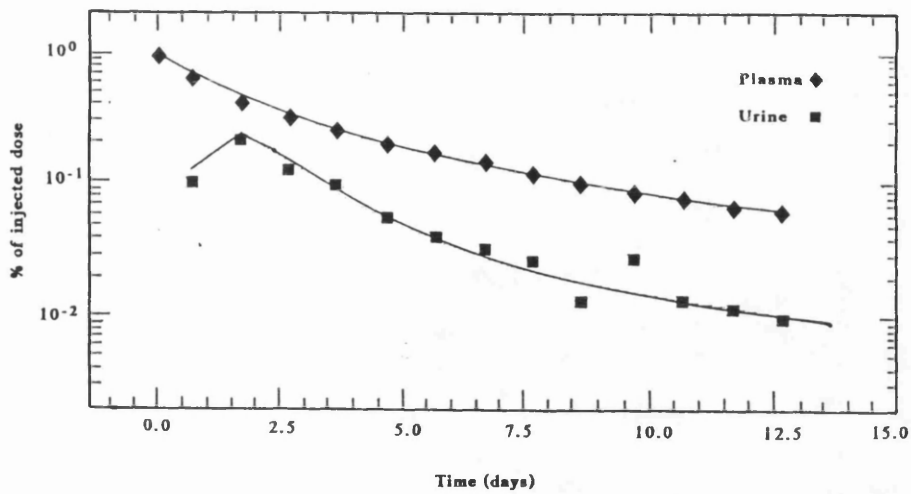


Figure 128 Cyclohexanedione Modified LDL

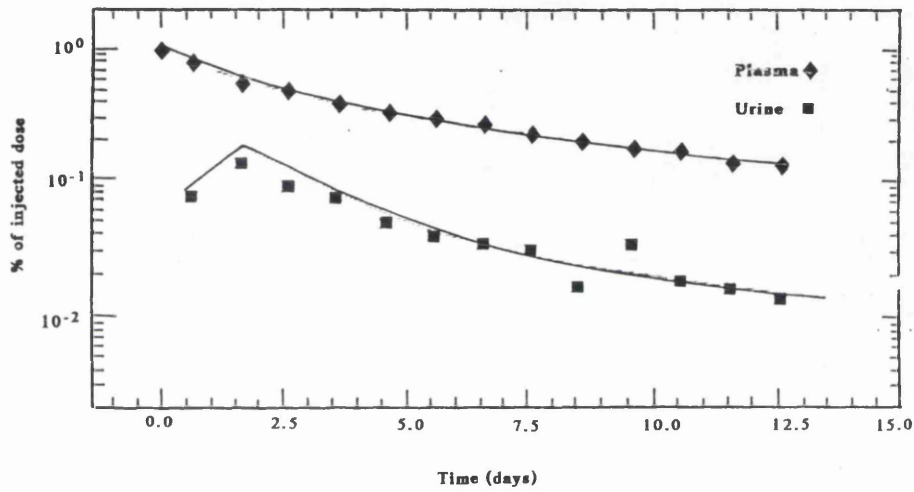
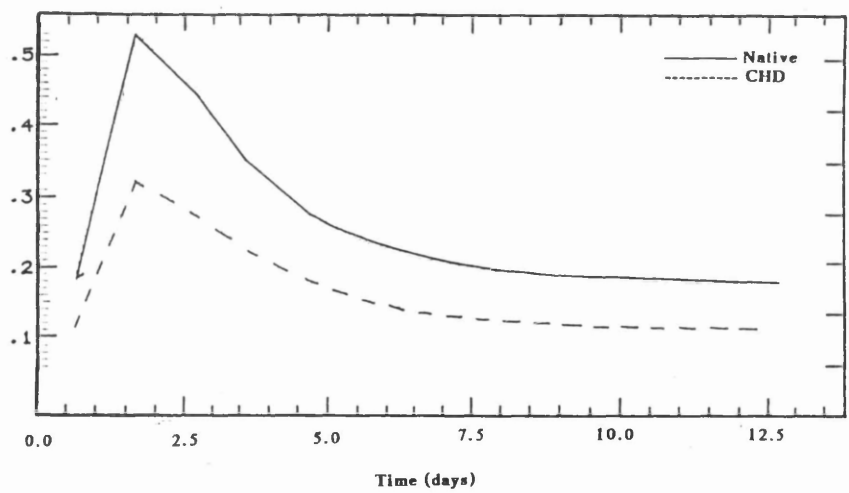


Figure 129 U/P Ratios



Figures A-127-129. CIP 05A. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 130 Native LDL

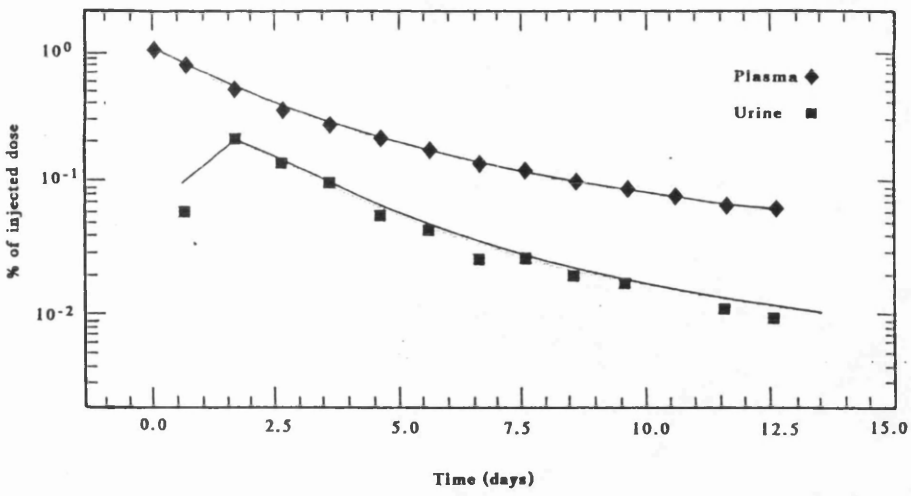


Figure 131 Cyclohexanedione Modified LDL

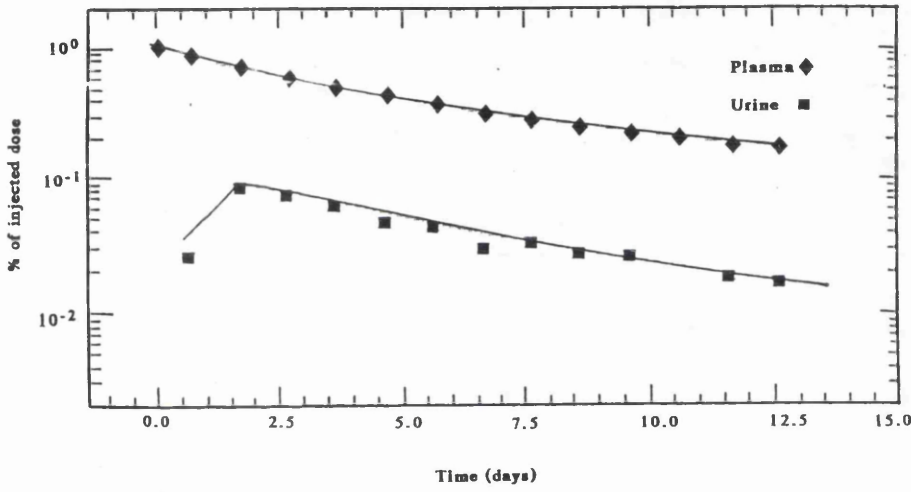
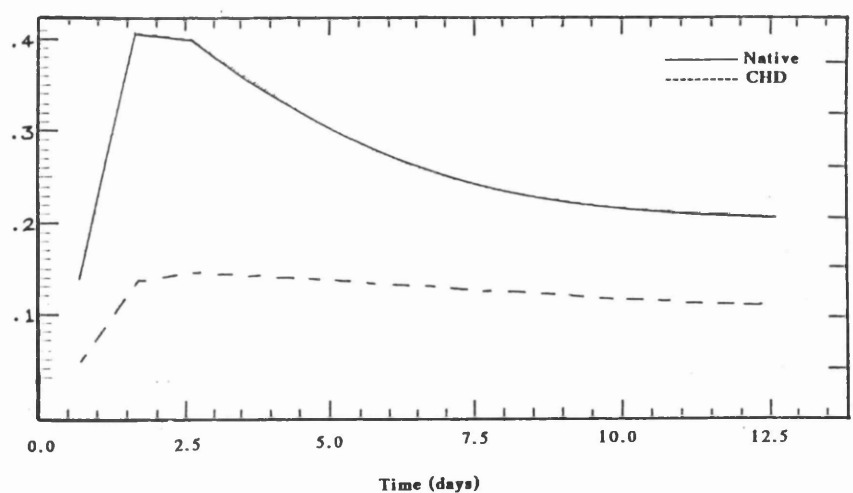


Figure 132 U/P Ratios



Figures A-130-132. CIP 05B. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 133 Native LDL

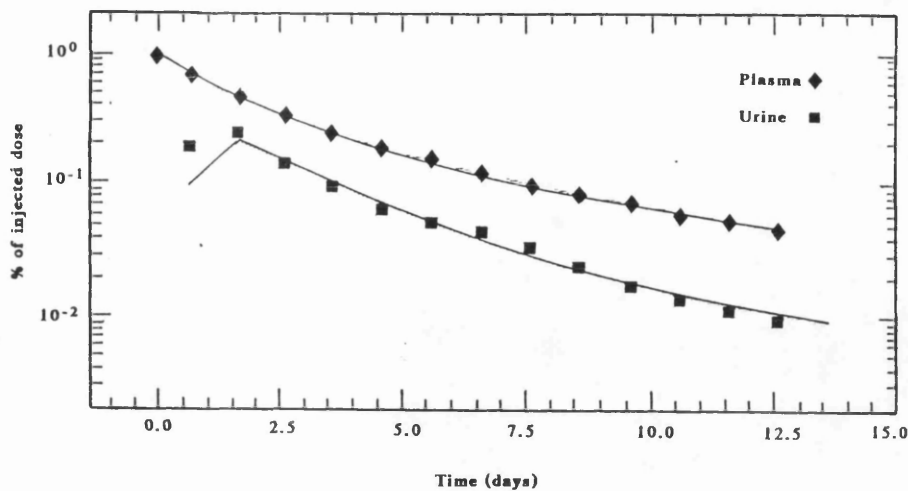


Figure 134 Cyclohexanedione Modified LDL

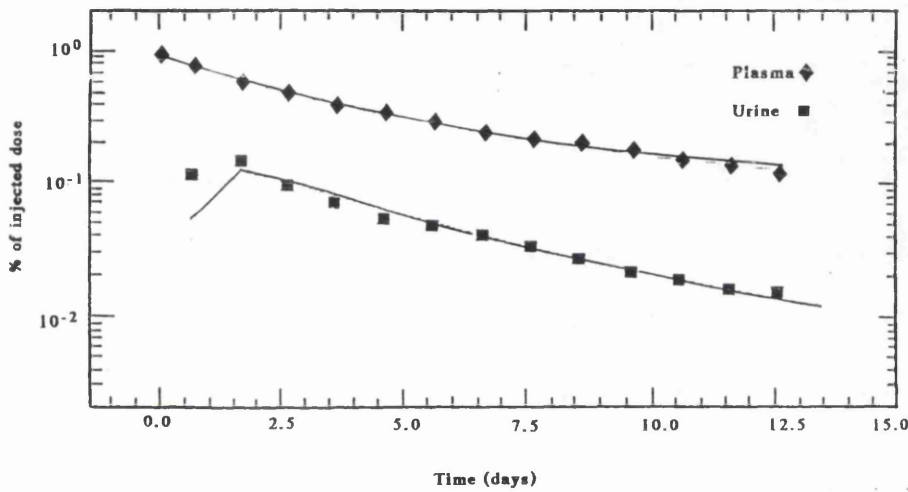
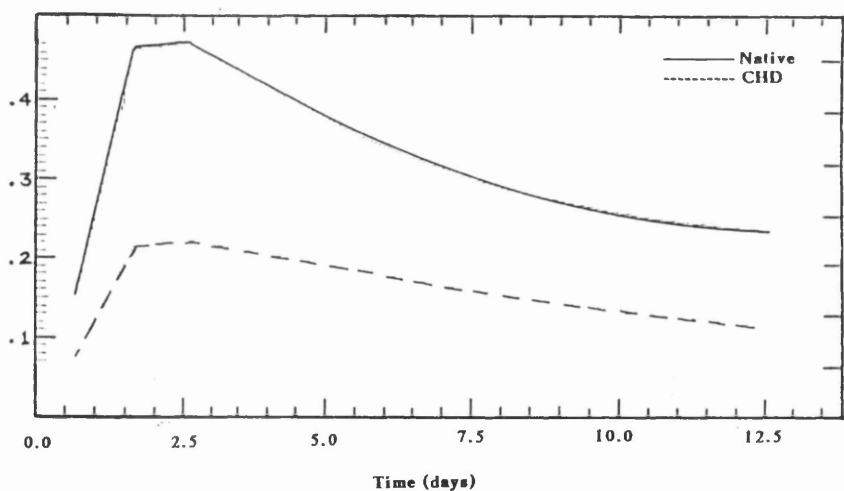


Figure 135 U/P Ratios



Figures A-133-135. CIP 06A. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure 136 Native LDL

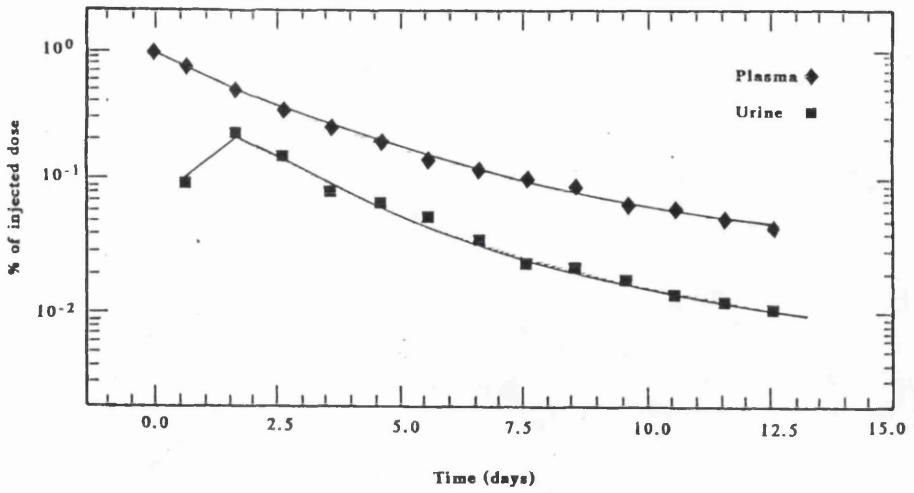


Figure 137 Cyclohexanedione Modified LDL

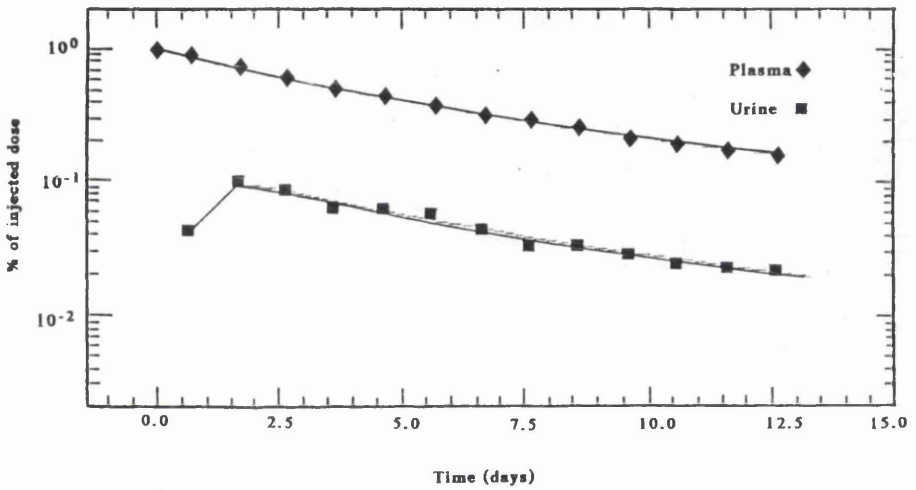
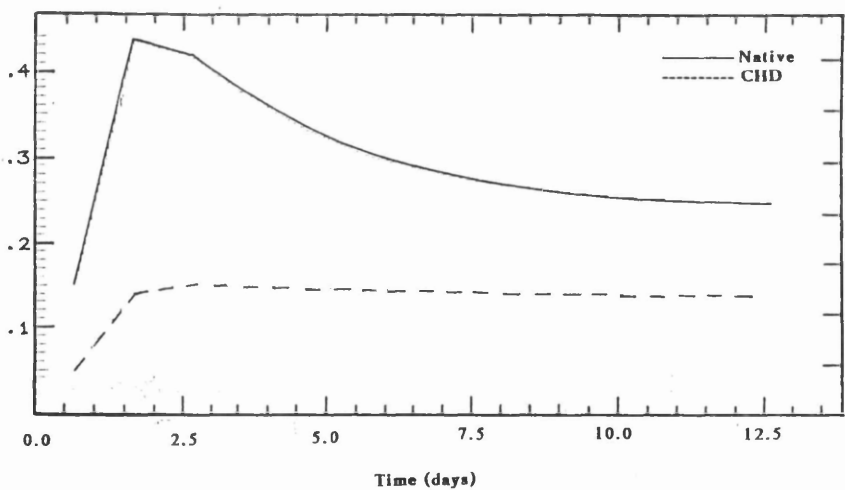


Figure 138 U/P Ratios



**Figures A-136-138.** CIP 06B. Plasma and urinary radioactivity decay curves following injection of [ $^{125}\text{I}$ ]-native apo-LDL and [ $^{131}\text{I}$ ]-cyclohexanedione modified apo-LDL.



Figure 139 Native LDL

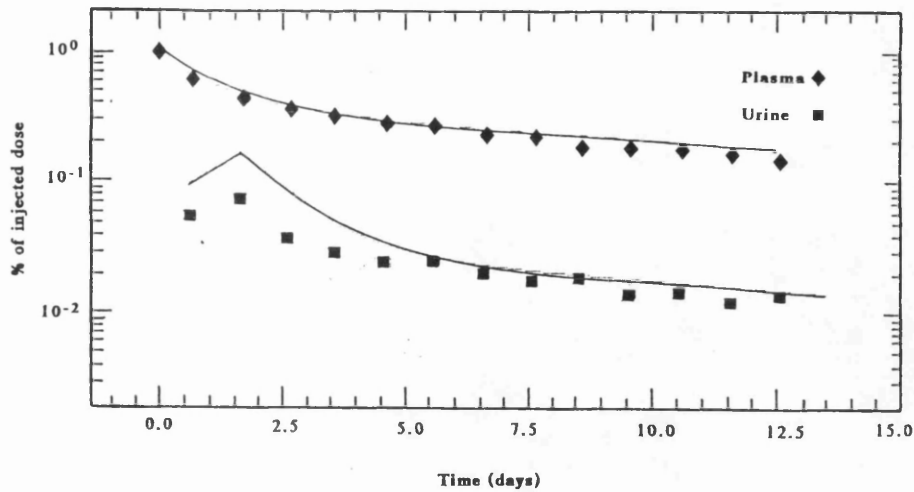


Figure 140 Cyclohexanedione Modified LDL

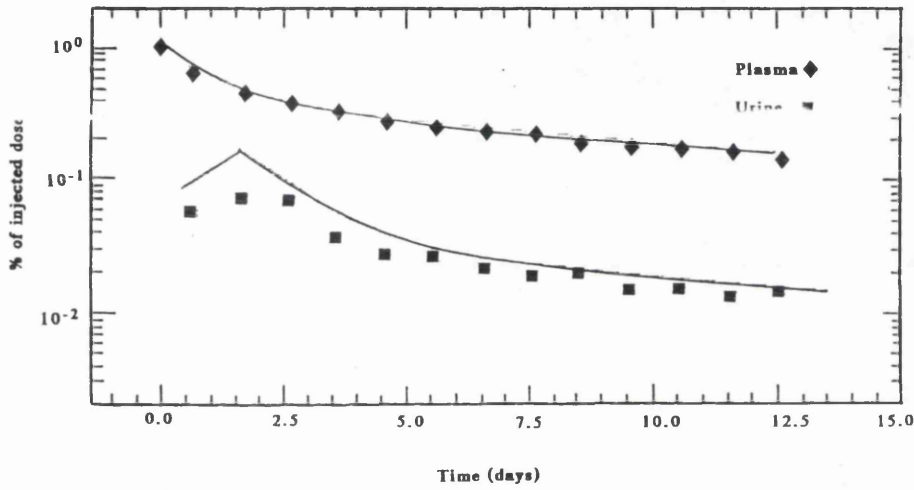
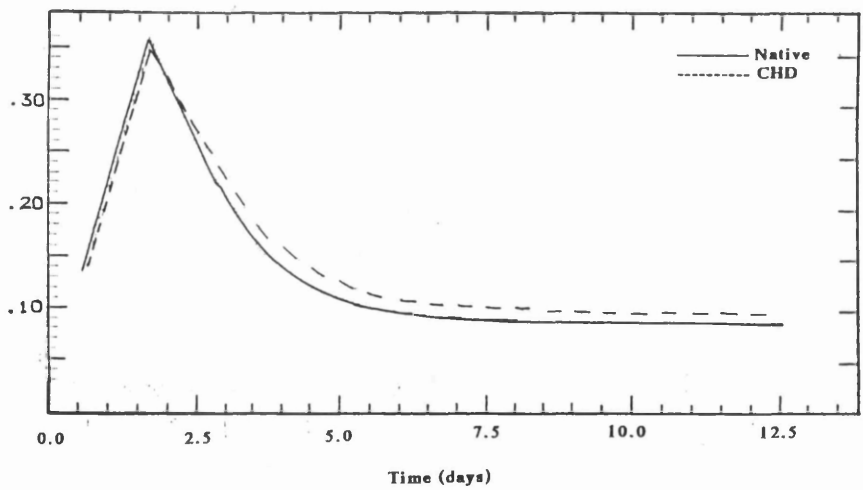


Figure 141 U/P Ratios



Figures A-139-141. CIP 07A. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure 142 Native LDL

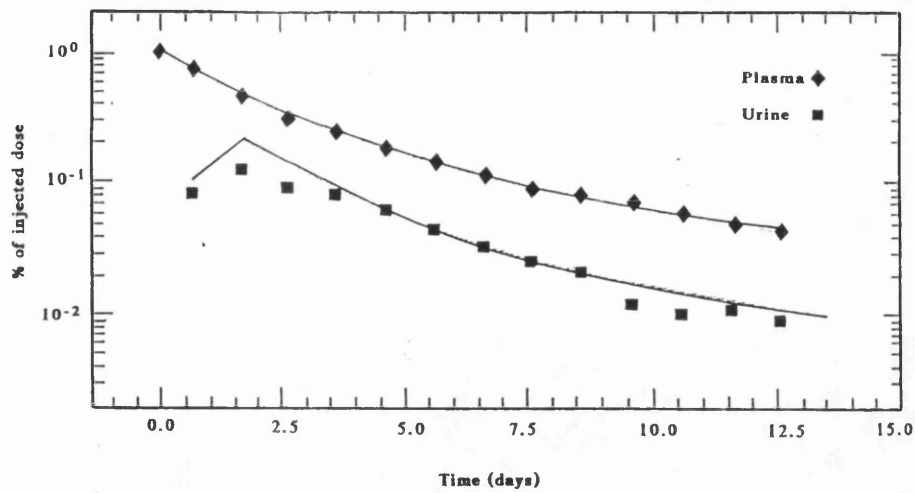


Figure 143 Cyclohexanedione Modified LDL

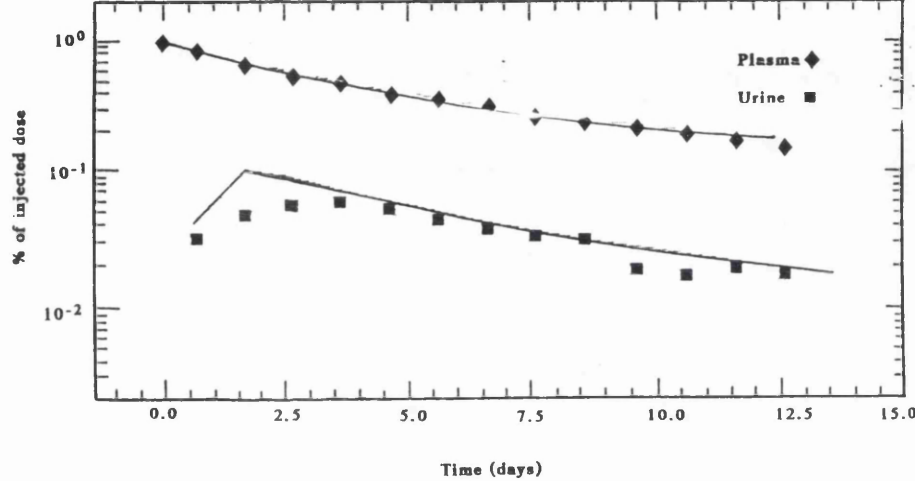
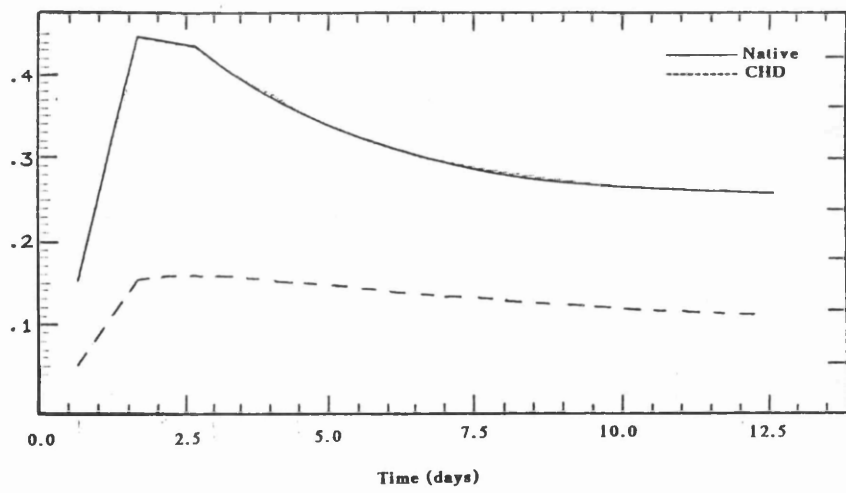


Figure 144 U/P Ratios



Figures A-142-144. CIP 07B. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

Figure 145 Native LDL

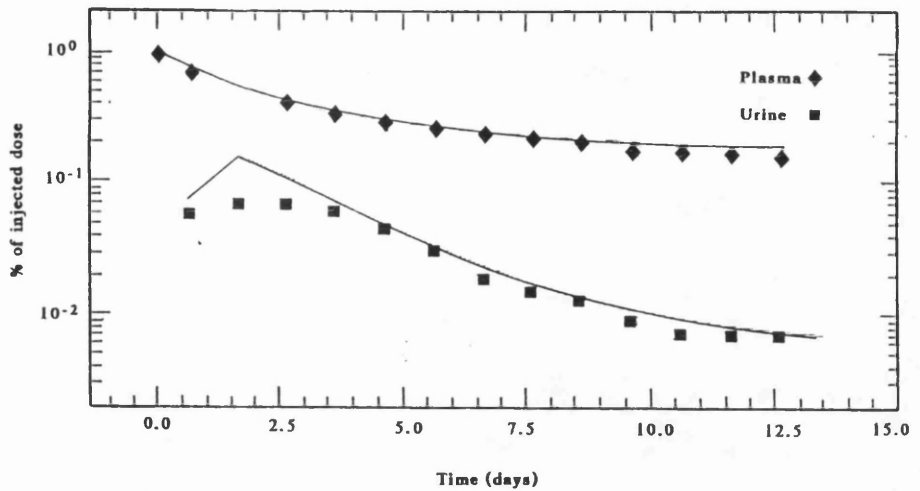


Figure 146 Cyclohexanedione Modified LDL

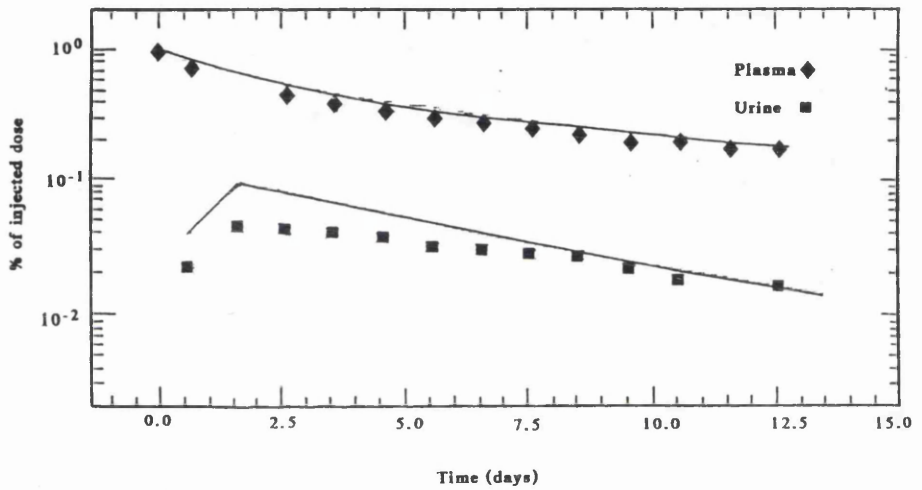
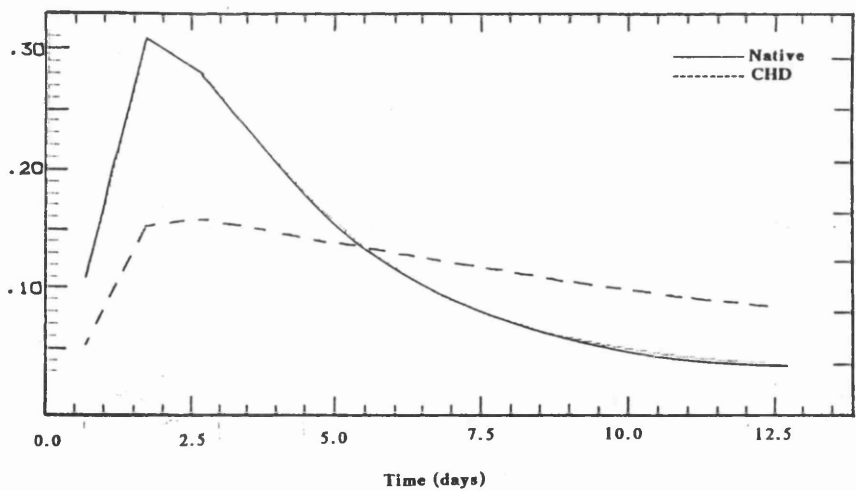


Figure 147 U/P Ratios



**Figures A-145-147.** CIP 09A. Plasma and urinary radioactivity decay curves following injection of [ $^{125}\text{I}$ ]-native apo-LDL and [ $^{131}\text{I}$ ]-cyclohexanedione modified apo-LDL.

Figure 148 Native LDL

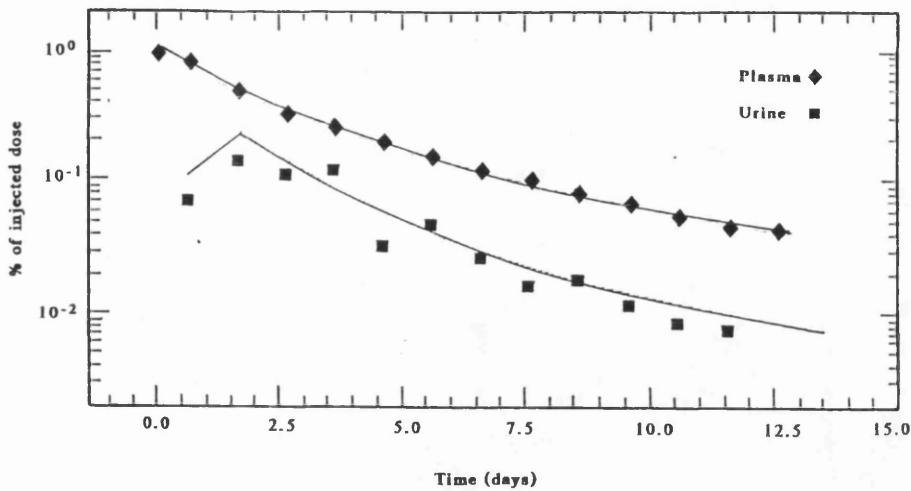


Figure 149 Cyclohexanedione Modified LDL

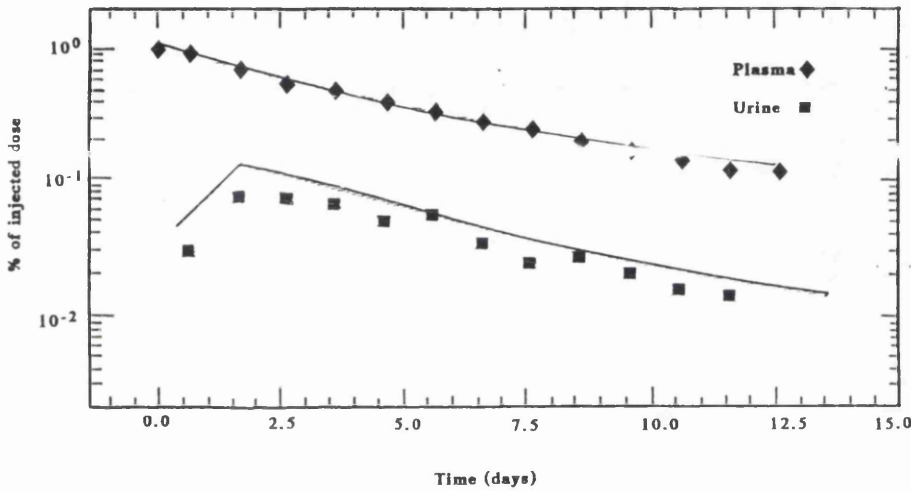
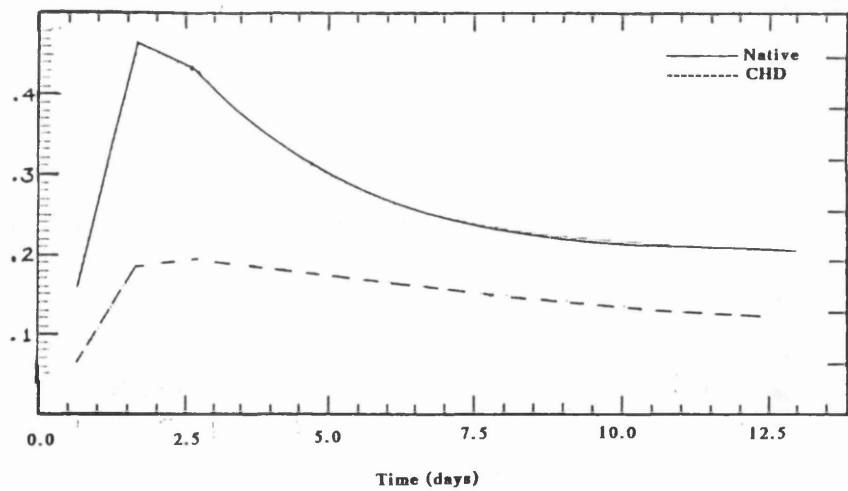


Figure 150 U/P Ratios



Figures A-148-150. CIP 09B. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 151 Native LDL

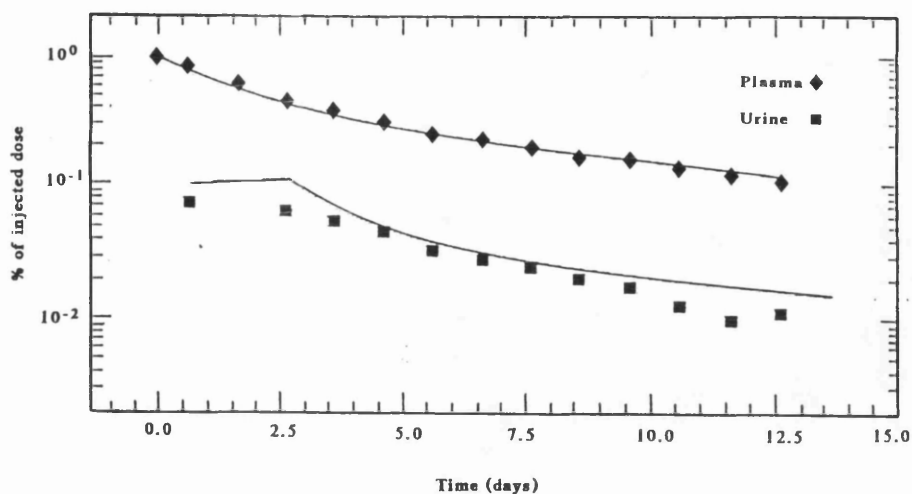


Figure 152 Cyclohexanedione Modified LDL

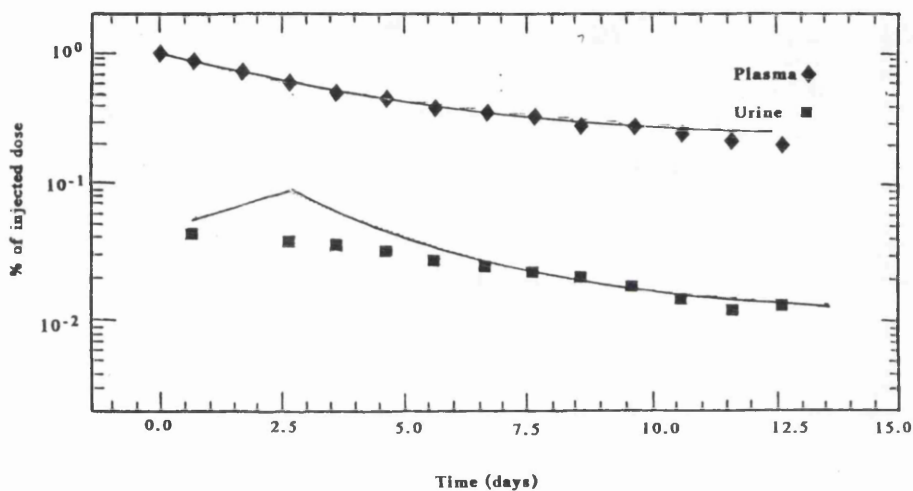
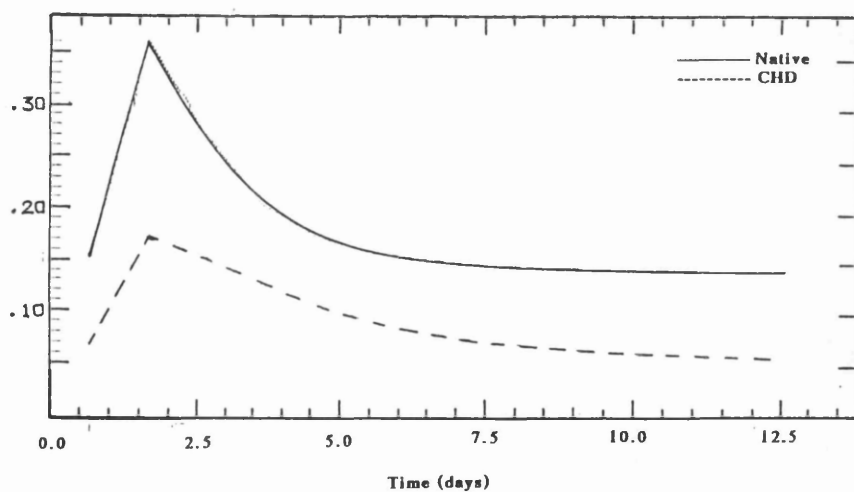


Figure 153 U/P Ratios



**Figures A-151-153.** CIP 10A. Plasma and urinary radioactivity decay curves following injection of [ $^{125}$ I]-native apo-LDL and [ $^{131}$ I]-cyclohexanedione modified apo-LDL.

Figure 154 Native LDL

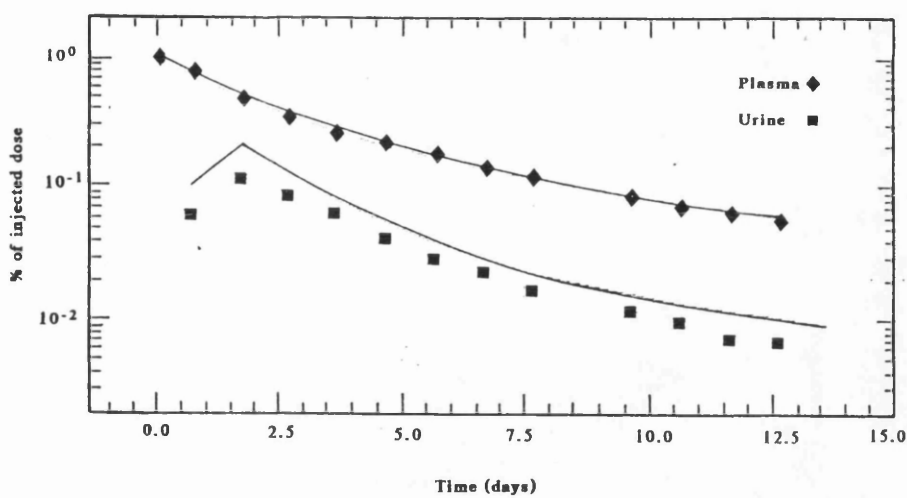


Figure 155 Cyclohexanedione Modified LDL

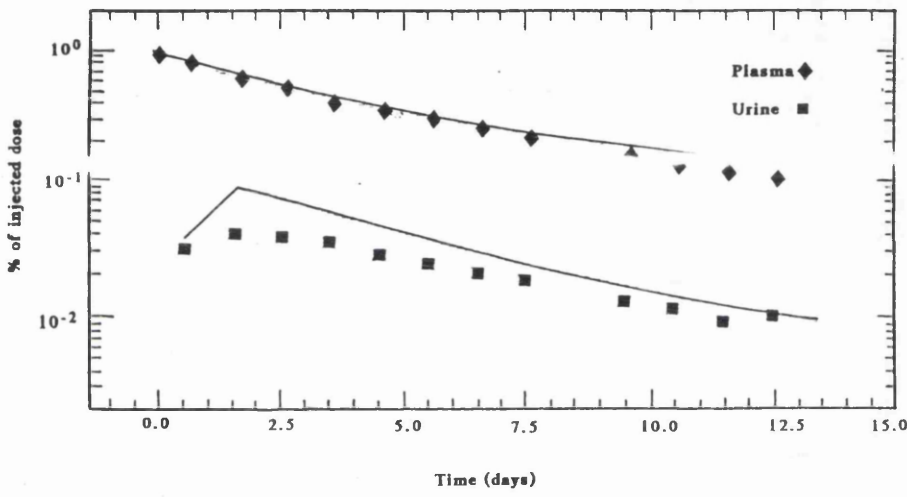
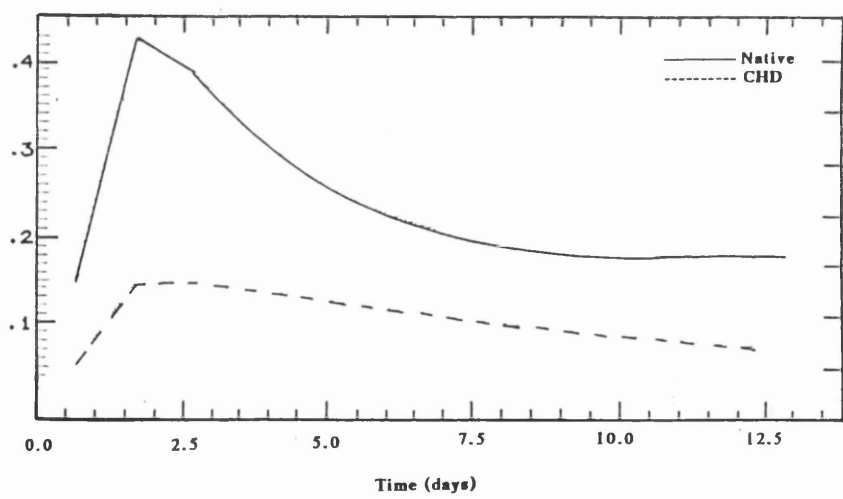


Figure 156 U/P Ratios



Figures A-154-156. CIP 10B. Plasma and urinary radioactivity decay curves following injection of [<sup>125</sup>I]-native apo-LDL and [<sup>131</sup>I]-cyclohexanedione modified apo-LDL.

*Glossary*

ACAT.....	Acyl Coenzyme A:cholesterol acyl transferase
ACR.....	Absolute Catabolic Rate
ALP.....	Atherogenic Lipoprotein Phenotype
Apo.....	Apolipoprotein
APX.....	Acipimox
BSA.....	Bovine Serum Albumin
cAMP.....	Cyclic Adenosine Monophosphate
CETP.....	Cholesteryl Ester Transfer Protein
CHD.....	Coronary Heart Disease
Ci.....	Curie
CIP.....	Ciprofibrate
CK.....	Creatine Kinase
CM.....	Chylomicron
COL.....	Colestipol
cpm.....	Counts Per Minute
CTX.....	Cerebrotendinous Xanthomatosis
d.....	Density
EDTA.....	Ethylenediaminetetra-acetate
FCR.....	Fractional Catabolic Rate
FEN.....	Fenofibrate
FH.....	Familial Hypercholesterolaemia
FSD.....	Fractional Standard Deviation
FSR.....	Fractional Synthetic Rate
GC-MS.....	Gas Chromatography-Mass Spectrometry
HDL.....	High Density Lipoprotein
HL.....	Hepatic Lipase
HMG CoA.....	3-hydroxy, 3-methylglutaryl Coenzyme A
IDL.....	Intermediate Density Lipoprotein
LCAT.....	Lecithin: cholesterol acyl transferase
LDH.....	Lactate Dehydrogenase
LDL.....	Low Density Lipoprotein
Lp(a).....	Lipoprotein (a)
LpL.....	Lipoprotein Lipase
LRC.....	Lipid Research Clinics
LRP.....	LDL Receptor Related Protein
MI.....	Myocardial Infarction
MM-LDL.....	Minimally Modified Low Density Lipoprotein
MVA.....	Mevalonic Acid
PEP.....	Phosphoenol pyruvate
PK.....	Pyruvate Kinase
rpm.....	Revolutions Per Minute
SAAM.....	Simulation, Analysis and Modeling
SDS.....	Sodium Dodecyl Sulphate
Sf.....	Svedberg Flotation Units
SIM.....	Simvastatin
TEAC.....	Triethylammonium carbonate
TMU.....	1,1,3,3-tetramethylurea
TRIS.....	Tris hydroxymethylamino methane
U/P.....	Urine/Plasma Ratio
VLDL.....	Very Low Density Lipoprotein
WHHL.....	Watanabe Heritable Hyperlipidaemic
WHO.....	World Health Organization